Disease defence in garden ants

by

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Doctor of Philosophy

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Abstract

Contagious diseases must transmit from infectious to susceptible hosts in order to reproduce. Whilst vectored pathogens can rely on intermediaries to find new hosts for them, many infectious pathogens require close contact or direct interaction between hosts for transmission. Hence, this means that conspecifics are often the main source of infection for most animals and so, in theory, animals should avoid conspecifics to reduce their risk of infection. Of course, in reality animals must interact with one another, as a bare minimum, to mate. However, being social provides many additional benefits and group living has become a taxonomically diverse and widespread trait. How then do social animals overcome the issue of increased disease?

Over the last few decades, the social insects (ants, termites and some bees and wasps) have become a model system for studying disease in social animals. On paper, a social insect colony should be particularly susceptible to disease, given that they often contain thousands of potential hosts that are closely related and frequently interact, as well as exhibiting stable environmental conditions that encourage microbial growth. Yet, disease outbreaks appear to be rare and attempts to eradicate pest species using pathogens have failed time and again. Evolutionary biologists investigating this observation have discovered that the reduced disease susceptibility in social insects is, in part, due to collectively performed disease defences of the workers. These defences act like a "social immune system" for the colony, resulting in a *per capita* decrease in disease, termed social immunity. Our understanding of social immunity, and its importance in relation to the immunological defences of each insect, continues to grow, but there remain many open questions.

In this thesis I have studied disease defence in garden ants. In the first data chapter, I use the invasive garden ant, *Lasius neglectus*, to investigate how colonies mitigate lethal infections and prevent them from spreading systemically. I find that ants have evolved 'destructive disinfection' – a behaviour that uses endogenously produced acidic poison to kill diseased brood and to prevent the pathogen from replicating. In the second experimental chapter, I continue to study the use of poison in invasive garden ant colonies, finding that it is sprayed prophylactically within the nest. However, this spraying has negative effects on developing pupae when they have had their cocoons artificially removed. Hence, I suggest that acidic nest sanitation may be maintaining larval cocoon spinning in this species. In the next experimental chapter, I investigated how colony founding black garden ant queens (*Lasius niger*) prevent disease when a co-foundress dies. I show that ant queens prophylactically perform undertaking behaviours, similar to those performed by the workers in mature nests. When a co-foundress was infected, these undertaking behaviours improved the survival of the healthy queen. In the final data chapter, I explored how immunocompetence (measured as antifungal activity) changes as incipient black garden ant colonies grow and mature, from the solitary queen phase to colonies with several hundred workers. Queen and worker antifungal activity varied throughout this time period, but despite social immunity, did not decrease as colonies matured.

In addition to the above data chapters, this thesis includes two co-authored reviews. In the first, we examine the state of the art in the field of social immunity and how it might develop in the future. In the second, we identify several challenges and open questions in the study of disease defence in animals. We highlight how social insects offer a unique model to tackle some of these problems, as disease defence can be studied from the cell to the society.

Acknowledgments

Moving to Vienna and conducting a PhD thesis has been extraordinary. I still sometimes cannot believe that I took the leap and I suspect I will look back on my time in Austria with astonishment. During my PhD I have learnt so many things about ants, science and schnitzel from so many wonderful people, and I am sure to forget a few in saying *Vielen Dank* to them here. Firstly though, I must thank the people back home, my friends and family, who were extremely supportive in sending me off with my bags packed, and for keeping in contact and welcoming me home with open arms. In particular, I must single out my Mum and Dad, who have always given me the freedom and encouragement to pursue my passions (who can forget the bedroom so full of stick insects we had to take the doors of the wardrobes to fit more in?). On that note, I must also thank my Grandparents, on both sides, for the same reason.

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About the Author

Christopher D. Pull completed a BSc in Zoology and an MSc in Biological Sciences Research at Royal Holloway University of London, before joining IST Austria in August 2012. His main research interests are animal behaviour and the evolution of sociality. Specifically, Christopher's research has focussed on disease in social animals, using ants and bees as study organisms. During his PhD, Christopher has presented his research at many international conferences, such as the European IUSSI conference in Helsinki, Finland in 2016, and attended several workshops, including the 2013 Tropical Behaviour, Ecology and Evolution field course in Gamboa, Panama and the 2014 Evolutionary Biology workshop in Guarda, Switzerland. In addition, Christopher created and organised a PhD course for international students studying social evolution, hosted by IST Austria in 2016.

List of Publications Appearing in Thesis

1. **Pull CD**, Ugelvig L V, Wiesenhofer F, Tragust S, Schmitt T, Brown MJF, Cremer S. 2017 Destructive disinfection of infected brood prevents systemic disease spread in ant colonies. *bioRxiv* (doi:10.1101/116657)
2. Cremer S, **Pull CD**, Fürst MA. 2018 Social immunity: emergence and evolution of colony-level disease protection. *Annu Rev Entomol* (doi:10.1146/annurev-ento-020117-043110)
3. **Pull CD**, Cremer S. 2017. Co-founding ant queens prevent disease by performing prophylactic undertaking behaviour. *BMC Evol Biol.* (in press)
4. Kennedy P, Baron G, Qiu B, Freitak D, Helanterä H, Hunt ER, Manfredini F, O'Shea-Wheller T, Patalano S, **Pull CD**, Sasaki T, Taylor D, Wyatt CDR, Sumner S. 2017 Deconstructing superorganisms and societies to address big questions in biology. *Trends Ecol Evol* (doi:10.1016/j.tree.2017.08.004)

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List of Abbreviations

GLM	General(ised) linear model
LMER	Linear mixed effects regression
GLMM	General(ised) linear mixed model
LR	Likelihood ratio test
CHC	Cuticular hydrocarbon
GC–MS	Gas chromatography–mass spectrometry
SEM	Scanning electron microscopy
perMANOVA	Permutational multivariate analysis of variance
KW	Kruskal–Wallace test
DAPC	Discriminate analysis of principal components

1. Introduction

1.1 Sociality and disease

Group living in animals confers many benefits, including enhanced protection against predators, better care of offspring and improved access to food [1,2]. Yet the costs of a social lifestyle are equally numerous and, in particular, the impact of pathogens (an infectious organism able to cause disease) has received considerable interest from biologists [1–6]. Social animals are expected to have experienced a disproportionately greater pathogen pressure – that is, increased prevalence, load and richness – during their evolution than solitary species, as a consequence of the higher level of within-group interactions that can lead to increased disease transmission [1,5]. It is therefore anticipated that social animals should invest more into controlling disease spread, as a result of adaptations that have evolved to mitigate this disease pressure.

Theoretically, the prediction sociality encourages disease transmission is well supported. Pathogens must successfully transmit to and infect new hosts in order to reproduce, and the rate at which a pathogen does so is known as its basic reproductive number (R_0) [7]. R_0 is simply the fitness of the pathogen, i.e. the number of new infections generated by an existing infection. It must be greater than 1 for a pathogen to spread in a population, but if R_0 falls below 1, i.e. each infection generates, on average, < 1 new infection, the disease will eventually die out [7]. R_0 is affected by several factors, but of particular importance are the size of the susceptible population (N) and the rate of transmission from host to host (β) [8]. In social animals, R_0 is expected to be higher because of a greater density of potential hosts (a larger N) and accelerated disease transmission, caused by more frequent contact, or social interactions, between infectious and susceptible hosts (a higher β; Figure 1) [8]. In addition, because social groups are often made up of families, there may be a higher within-group probability of infection success [9]. Since closely related individuals (e.g. siblings) share, on average, a greater proportion of genes than random members of the population, social groups made up of families will be more genetically similar [10]. As some pathogen genotypes are more likely to infect certain host genotypes, reduced genetic diversity within family groups may promote infection success, as a pathogen able to infect one group member will likely to able to infect a large proportion of the others [3]. Conversely, in a randomly mixed population of many genotypes, a pathogen will regularly encounter hosts that it cannot infect, thus reducing R_0 [11–13].

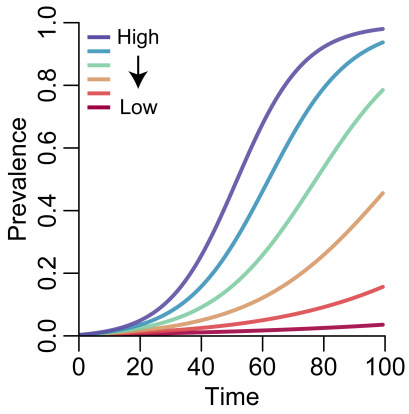

Figure 1. An increase in social interactions (causing a higher rate of transmission, β) that have a fixed probability of instigating disease transmission (high-low; per host/unit of time) leads to a faster spread and higher proportion of infected hosts (disease prevalence).

Empirically, several studies have demonstrated that the above expectation appears to hold true, with both pathogen load and richness increasing with group size [14,15]. However, this correlation is dependent on the mode of disease transmission: pathogens that require host-host contact have greater impact in social groups, whilst those that are transmitted via vectors (e.g. by mosquitos) affect social and solitary animals equally. This makes sense because vectored pathogens are less likely to rely on social interactions for transmission and can be

carried across greater distances to infect hosts that are dispersed. In some cases, however, the correlation between group size and pathogen burden is weak or missing altogether [14–16]. Furthermore, social animals can be more resilient to disease than solitary individuals [17]. These observations have led to a new hypothesis: animals living in groups may be under selection to evolve adaptations that offset the increased cost of disease, by investing more into controlling the disease pressure they experience [3–6]. Such adaptations may range from increasing the genetic diversity within the social group [18,19] to behavioural defences that prevent pathogen transmission and infection [17,20]. These adaptations will constitute an essential component of group living and may explain how sociality is able to persist despite an increase in disease transmission within groups [5].

One disease-reducing behaviour that has been observed in animals as diverse as lobsters and mandrills, is infection avoidance [20]. By detecting disease-associated cues – typically chemical signatures of infection – healthy animals can avoid mixing or mating with sick conspecifics [21–26]. Additionally, animals may still associate with infectious individuals, but modulate how they interact to reduce the risk of transmission [25]. The emotion disgust, expressed by humans in response to images connoting the presence of pathogens, has also been linked to infection avoidance behaviour, suggesting that psychological mechanisms of infection avoidance are also important in social animals [27]. These adaptations, among others, reduce the chances of pathogens successfully transmitting to new hosts and can, therefore, curb disease spread by decreasing the magnitude of R_0 [28]. Such behaviours help social animals to resist diseases, but sociality may also offer alternative strategies that are not possible in solitary species [5]. More recently, the role of tolerance – alleviating the symptoms of disease, rather than clearing the pathogen – has been investigated in animals [29,30]. Early results suggest that social animals may better tolerate the negative impact of pathogens [5,31]. This is because the benefits associated with sociality (increased access to resources, in particular food and increased predator vigilance) may make it easier for social animals to buffer the impacts of pathogens, likely increasing resilience [31].

To summarise, social animals are expected to have experienced a greater selective pressure from pathogens over the course of their evolutionary history than solitary species. As a consequence of this pathogen-induced selection acting on group-living animals, they should have evolved adaptations that counterbalance this pressure. In the next section, I will discuss how complex these adaptations can become when sociality evolves beyond simple group living to complex, eusocial societies and superorganisms.

1.2 Social immunity

The evolution of life has been punctuated by several major evolutionary transitions, where previously free-living and independently replicating individuals cooperated to form new, more complex life forms (Figure 2) [32,33]. For example, individual genes formed genomes, individual prokaryotes formed eukaryotes and individual cells formed multicellular organisms. As the new life forms replicate together as a whole, the level at which natural selection acts shifts, from the separate individuals to the new cooperating entity. This also means that the fate and interests of the cooperating individuals making up the new life form are

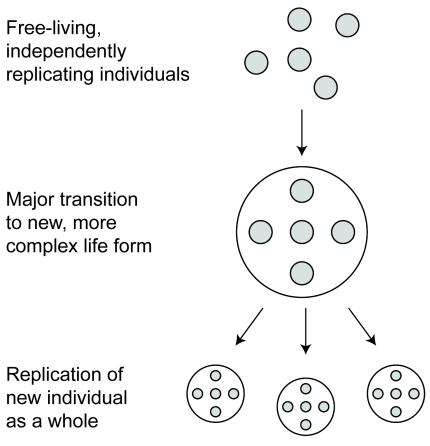

Figure 2. Schematic of a major evolutionary transition.

intimately linked, and they become mutually dependent on one another for survival and reproduction.

Another major evolutionary transition occurred relatively recently in the eusocial insects – the social wasps, social bees, ants and termites. These insects form colonies that exhibit reproductive division of labour, where some individuals – known as queens and males – are specialised for reproduction, whilst the other insects remain sterile [34]. Instead of dispersing and reproducing, the sterile individuals raise the offspring of the reproducers and are hence termed workers. Because colonies are typically single families, individuals in a colony are closely related [35–37]. By helping the queens to reproduce, the workers therefore gain indirect fitness by ensuring copies of their genes, present in the queen's offspring, are passed on into the next generation [36,38]. By efficiently dividing labour this way, the queens and workers become mutually dependent on one another for survival and reproduction. Crucially, when these roles become morphologically fixed so that the workers remain unmated for their entire life (i.e. can never become queens), they are considered to have undergone a major evolutionary transition (i.e. vespine wasps, corbiculate bees, ants and higher termites) [39–41]. The new, more complex life form that arises is called a "superorganism", which is made up of many cooperating organisms [39–41].

For the same reasons discussed in the previous section, eusocial insects are expected to suffer more from disease than solitary insects [3,42,43]. They may even be more susceptible than other social animals, due in part to the especially low genetic diversity in the colonies of some species [3,6]. Furthermore, the extremely high density of hosts and the frequency at which they engage in intimate interactions (e.g. food sharing through regurgitation) [44,45], coupled with the high microbial loads in their environment and stable nest conditions that encourage microbial growth [46–48], should, in theory, mean that social insects suffer frequent, devastating epizootics. In the domesticated honeybee, the major loss of colonies has been partially attributed to emergent infectious diseases, alongside other stressors, demonstrating the damage pathogens can cause in susceptible populations. However, the evidence for regular, natural disease outbreaks in social insects is rare, and decades of research attempting to eradicate pest termite species using biological controls, such as infectious fungi, have failed [49]. Hence, as in group-living animals, the superorganismal insects appear to have evolved mechanisms to overcome the increased risk of disease present in their colonies.

As in the other major transitions, the emergence of superorganismality required adaptations to maintain the functioning of the new life form [50]. Firstly, conflicts of interest need to be solved so that cooperation is preserved and cannot be exploited from within by selfish elements. Secondly, the new life form needs to be protected from external threats that would diminish the benefits gained by cooperation, namely, pathogens. Dealing with invading organisms requires the recognition of self from non-self, and over time, this simple distinction has evolved into extremely effective defence against disease. For example, in multicellular organisms, the immune system detects pathogens, prevents their establishment and can even recognise the same pathogen to more efficiently eliminate it in the future. In superorganisms, an analogous defence system has evolved, where workers perform collective behaviours that reduce the chances of infection, as well as the proliferation and transmission of successful infections [17,43,51]. Like the immune system, these behaviours are layered and typically require the concerted actions of the workers [17,43,51]. Overall, these defences result in a reduced risk of disease for the colony, termed "social immunity" [17].

1.3 Study organisms

Figure 3. An invasive garden ant colony in the laboratory.

1.3.1 The invasive garden ant *Lasius neglectus*

Over the past decade, the invasive garden ant, *Lasius neglectus*, has been developed as a model system for studying social immunity [52–56]. As its name implies, *L. neglectus* was only discovered relatively recently in, Budapest, Hungary [57]. However, it is also found living in Asia Minor where its non-invasive sister species, *L. turcicus*, is also present, making it likely that *L. neglectus* was introduced accidentally to Europe [58–60]. It has since spread and is now found over large areas of Europe [58,60]. Unlike other invasive ant species, *L. neglectus* can survive colder weather and extended periods of frost [58]. The continued spread of *L. neglectus* therefore seems likely and it has now started spreading within the United Kingdom [61], though in some areas supercolonies have contracted or collapsed [62]. Like other invasive ant species, it exhibits the supercolonial syndrome: colonies comprise large networks of cooperating nests that contain many, non-dispersing queens that mate within the nest [63]. Workers outcompete native ant species [64] and collect honeydew from aphids *en mass*, leading to increased tree damage in infested areas [65]. As *L. neglectus* inhabits disturbed urban areas, such as parks and gardens, large numbers of queens, workers and brood can be collected relatively easily in the field. Moreover, as queens are produced and mate in the laboratory, colonies can be maintained in captivity over successive years, making them ideal models for laboratory studies.

1.3.2 The black garden ant *Lasius niger*

Figure 4. Founding colonies of black garden ant queens.

The black garden ant, *L. niger,* has a lifestyle more representative of typical ants than that of *L. neglectus*. Colonies contain several thousand workers but only a single queen, and are hostile to all other neighbouring colonies [66]. Sexuals (males and queens) are produced annually and leave the nest to engage in large, nuptial flights [67–69]. These flights are probably synchronised with other colonies in the population through environmental cues [70]. After mating, the males die and the queens are left to found a colony alone, without the assistance of workers. Upon landing, the queens shed their wings and begin scurrying about, investigating potential areas to dig a nest. Once a queen finds a suitable location – often under a stone – she will begin excavating a nest chamber. At this stage, other queens may join her to found a colony together, which is known as pleometrosis [70]. In *L. niger*, co-founding occurs in 18% of cases with a median of two queens per nest [66]. The exact cause

of co-founding is unknown, but possibilities include improved queen survival, benefits from a larger initial number of workers or simply overcrowding resulting from a lack of nest sites [71–76]. Once sealed inside her subterranean bunker, the queen does not forage and must raise her first workers by metabolising body fat, wing muscle and even parts of her brain associated with vision, given that she will never again fly or see daylight [77,78]. Many queens die from exhaustion, predators and pathogens during this stage [70,79–81]. In addition, queens in colonies started by more than one individual will fight to the death once the first workers emerge, as cooperation is no longer beneficial [66,70,82]. Those queens that do survive can live many years, with reports of some even surviving multiple decades [83]. During this time, the queen lays tens of thousands of eggs and colonies can grow very large. However, once a queen dies she is not replaced and the colony eventually perishes without the regular replacement workers. Because queens are produced in such great numbers and readily initiate colonies in the laboratory, *L. niger* is an ideal species for longitudinal research investigating how traits emerge and develop as colonies grow and mature.

1.3.3 The entomopathogenic fungus *Metarhizium brunneum*

The ascomycete genus *Metarhizium* causes the green muscardine disease in insects and is commonly used as a biological insecticide [84]. Although some species have restricted host ranges, *Metarhizium* is typically considered a generalist pathogen, given some species are able to infect hundreds of insect species, from at least seven orders. It is also extremely widespread and has been isolated from all continents except Antarctica [85]. All species follow approximately the same lifecycle: insects acquire the soil-borne, infectious conidiospores from the environment, which initially attach loosely to the insect's cuticle. If conditions are sufficiently humid, the conidiospore will swell and produce a germ tube that gives rise to a specialised infection structure called an appressorium [84]. Penetration into the hemocoel is achieved through the production of enzymes, including lipases, chitinases etc. that breakdown the cuticle. Once the fungus penetrates the cuticle, it buds to produce single cells, known as blastospores, which spread throughout the insect's body via the hemolymph. The blastospores consume sugars in the hemolymph and produce destruxins, toxins that have a wide range of biological effects on the host, including suppressing the immune system and killing cells [86]. Hence, destruxins and the colonisation of vital organs, such as the fat body, are the primary cause of host death in *Metarhizium* infections [85]. Following host death, the fungus switches to a saprophytic stage, producing mycelia that eventually grow out of corpse through the intersegmental regions. Soon after the mycelia sporulate to produce new, asexual conidiospores at the tips of structures called phialides [84]. *Metarhizium* is therefore semelparous and reproduces only after host death. *Metarhizium* can produce millions of new conidiospores per corpse, which can go on to infect new hosts. The relatively small conidiospores can be found in high abundances in the environment [48] and the chances of successful infection increase with the number of spores encountered within a certain time

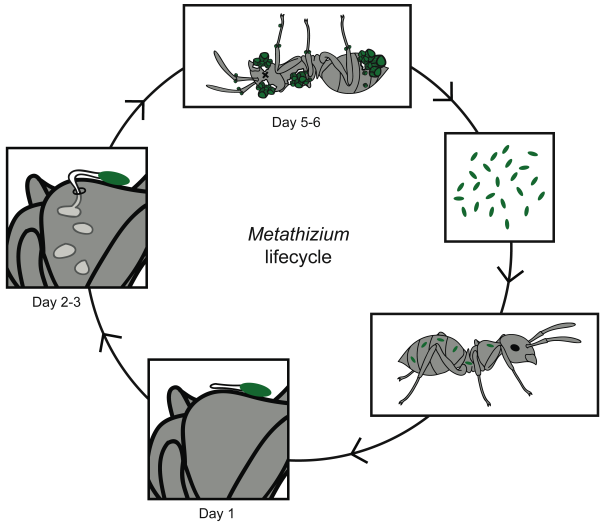

Figure 5. A schematic diagram of the lifecycle of *Metarhizium*

period, however, even very low dosages can cause lethal infections [87–89]. Throughout this thesis, I used the species *M. brunneum* (strain: KVL-03-143, MA275) as a model pathogen, which is easily cultured on agar dishes and can be stored in glycerol at – 80ºC for long durations [90]. Infectious conidiospores are harvested from fully sporulating plates and suspended in 0.05% Triton-X, which can then be applied directly to hosts by topical application at specific concentrations and dosages [90].

1.4 Thesis aims and outline

In this thesis, I aimed to (i) gain a greater understanding of how ants respond to pathogens when initial social immunity defences are unable to prevent an infection and (ii) how immunity develops as a colony grows. Interest in the field of social immunity and what we can learn from studying disease in social insects is continually increasing and new tools are offering fresh insights into how, for example, colony organisation affects disease transmission in colonies. Moreover, with the wealth of information we now have, it is becoming possible and necessary to place this information into a larger ecological and evolutionary perspective.

To that end, the following **second chapter** synthesises the current state of the art for social immunity in a review written by S Cremer, MA Fürst and myself. In this review, we summarise the history of the field of social immunity and the current literature. In an attempt to place social immunity to a broader eco-evolutionary framework, we discuss how social immunity may have evolved and its implications for host-pathogen interactions. Finally, we suggest areas where the field requires more expansion and how modern tools, such as tracking and sensitive molecular techniques can pave the way for a comprehensive understanding of social immunity and the pathogens that infect insects.

In the **third chapter**, I describe a novel, multicomponent behaviour performed by invasive garden ants that prevents intra-colonial disease transmission. In this study, I exposed ant pupae to the fungal pathogen *Metarhizium brunneum* and studied how the ants respond when sanitary care fails to prevent infection. I found that the ants remove infected pupae from their cocoons, bite holes in their cuticle, and administer acidic poison through the resulting wounds. In a series of experiments, I show that this behaviour, termed destructive disinfection, is triggered by olfactory cues emanating from the pupae and that all three behaviours – cocoon removal, biting and poison spraying – are necessary and work synergistically to prevent the pathogen replicating and transmitting to new hosts. This behaviour could have important consequences for host-pathogen interactions between ants and common soil pathogens, given that the fitness of the fungus is reduced to zero.

In the **fourth chapter**, I show that invasive garden ants spray their poison prophylactically in the nest and that this can have negative effects on the survival of the brood. Specifically, the delicate, metamorphosing pupae are damaged by poison if they are artificially removed from their cocoons. However, under normal conditions, cocooned pupae are protected because the silken, hydrophobic cocoon prevents the poison coming into the contact with the pupae inside. As cocoon spinning is costly and has been lost in other species that do not spray poison, this data suggests that the poison may be selecting for the maintenance of the cocoon in this species. Moreover, the presence of the cocoon may relax constraints on of toxicity of the poison and prevent 'social immunopathology'.

In the **fifth chapter**, I study infection avoidance behaviour in the colony-founding queens of the black garden ant. In this species, queens can found colonies alone or with other, usually unrelated, conspecifics. However, many queens contract infections and die during this period and it is unknown how co-founding queens then deal with these corpses and if they can prevent disease transmission. To address this problem, I performed two experiments to study (i) how pathogen exposure affects the colony founding choice of queens and (ii) whether the queens perform infection avoidance behaviours if a co-founder dies. I show that queens co-found with both sham-treated and pathogen-exposed queens. However, if a co-foundress dies, the surviving queen performs undertaking behaviours (corpse-induced responses) equally towards both infected and non-infected corpses. These behaviours occur before the corpse becomes infectious and reduces the risk for the queen to contract the disease. Hence, undertaking behaviour may play an important role in colony-founding success of queens.

The **sixth** chapter aims to understand how the immunocompetence of incipient black garden ant colonies changes as the colony grows and develops. To that end, I collected mated founding queens and let them establish colonies in the laboratory. I then tested the immunocompetence of the queens at specific time points, starting colony foundation by single queens to growing colonies with hundreds of workers. In addition, I looked at how immunocompetence differs between the first workers produced by the queens and those in older colonies. I present preliminary results from this experiment that show immunocompetence in colony founding queens is dynamic and influenced by brood production and colony size.

In the final, **seventh chapter**, D Freitak and I identify several open questions in the study of immunity and disease defence. We highlight how social insects offer valuable model systems with which to try and answer these questions. Namely, social insects offer tractable models to study immunity at many levels, from cellular processes to complete, interacting societies.

2. Social immunity: emergence and evolution of colony-level disease protection

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Invited (SC) review – under review at *Annual Review of Entomology*

Abstract
Social insect colonies have evolved many collectively performed adaptations that reduce the impact of infectious disease and maximize their fitness. This colony-level protection is termed social immunity and enhances the health and survival of colony. In this review, we address how social immunity emerges from its mechanistic components to produce colony-level disease avoidance, resistance and tolerance. To understand the evolutionary causes and consequences of social immunity, we highlight the need for studies evaluating the effects of social immunity on colony fitness. We discuss the role of host life history and ecology on predicted eco-evolutionary dynamics, which differ between the social insect lineages. Throughout the review we highlight current gaps in our knowledge and promising avenues for future research, which we hope will bring us closer to an integrated understanding of socio-eco-evo-immunology.

Keywords
host-pathogen dynamics; superorganism; behavioural disease defence; collective behaviour; resistance; tolerance

Author contributions
Conceived review: SC, CDP, MF
Literature review: SC, CDP, MF
Figure design: SC, CDP, MF
Wrote paper: SC, CDP, MF

Sections in detail
Introduction: SC, CDP (30%), MF
Social immunity: SC, CDP (60%), MF
Eco-evolutionary dynamics of social insect host-pathogen interactions: SC, CDP (20%), MF
Conclusions and outlook: SC, CDP (30%), MF
Sidebars: SC, CDP (50%), MF
Figures: SC, CDP (20%), MF

2.1 Introduction

The eusocial wasps, bees, ants and termites [91] are evolutionarily and ecologically successful, having persisted and diversified over millions of years to now inhabit almost every ecosystem on the globe [92,93]. The success of the social insects is due in no small part to their social lifestyles, which, among other benefits, makes it easier for them to colonize new habitats and fill ecological niches [93]. However, sociality is not without its drawbacks [1,4]. For a long time, a social lifestyle has been thought to increase the risk of disease [1,4]. This is because pathogens – i.e. disease causing agents like fungi, bacteria and viruses – exploiting social interaction networks within animal groups can spread more easily between infectious and susceptible individuals, than in solitary species [1,4,42].

In 1987, Hamilton pointed out that this problem is exacerbated in social insects by the low genetic diversity in their colonies. He reasoned that because they are typically single families, a pathogen able to infect one insect genotype should be able to spread and infect all others [3]. Later, Schmid-Hempel identified several other aspects of social insect biology, such as living within homeostatic nests in pathogen-rich environments, which further increase the risk of disease outbreaks [42]. At the same time, Schmid-Hempel developed a framework for how sociality affects host-pathogen interactions and evolutionary dynamics in social insects [42].

Despite these apparent vulnerabilities, social insects seem to cope with diseases remarkably well, and epizootics killing colonies are rare [94]. To understand this observation from an ecological perspective, Boomsma *et al.* assessed how the life history and ecology of the different social insect linages affects the pathogenic pressure they experience [43]. Their major conclusions were that, as hosts, ants and termites share many similarities, whilst the same is true of bees and wasps. These similarities should predispose them to a comparable set of pathogens with equivalent transmission routes, and, in turn, select for comparable host defences. Based on a growing number of studies, Boomsma *et al.* outlined the behavioural and physiological adaptations that insect colonies express, in addition to the immunological defences of colony members, which reduce disease susceptibility at both the individual and colony-level.

In 2007, Cremer *et al.* introduced the term "social immunity" to describe the colony-level disease protection that is achieved through the collective defences of colony members [17]. These defences were categorized into behavioural, physiological and organizational components, which function jointly to prevent the uptake, establishment and replication of pathogens in the colony. Building on the pioneering work of Schmid-Hempel and Naug & Camazine [42,45,95], Cremer *et al.* highlighted the role of colony organization as a unique feature of social immunity, which should be under selection to prevent disease spread within colonies. In 2009, Cremer and Sixt conceptualized the remarkable number of similarities between social immunity and the organismal immunity of a multicellular body, arguing that, in effect, social immunity functions as the immune system of the colony (see 2.1) [51].

Over the following decade, studies on social immunity have steadily increased. In addition to earlier work using mostly honeybees [96,97] and bumblebees [98], several well-established host models have been developed for the termites [99–101] and ants [52,102–106]. The wasps, however, remain underrepresented. In this review, our aim is to cover the expansion of studies on social immunity and recent advances in the field. We start by discussing the evolution of social immunity and how it differs from other group-level disease defences. We

then look at how social immunity emerges from its components to provide colony-level protection by avoidance, resistance or tolerance. In the third section, we consider how social immunity affects eco-evolutionary dynamics between social insects and their pathogens. Finally, we conclude by highlighting current challenges and prospective avenues for future social immunity research.

2.2 Social immunity

2.2.1 The Immune System of the Colony

Social immunity results from the collectively performed defences of colony members [17,42]. It is composed of behavioural, physiological and organizational defences that are carried out by workers together or towards one another [17]. Social immunity measures often initially involve nest hygiene and sanitary care behaviours that prevent or reduce the disease risk of colony members, for example, when they are contaminated with a pathogen[55,101,107]. However, if this first line of defence fails, selection may then favour the elimination of the infected individual to protect the colony [56,105,108–111]. Social immunity can therefore be characterized as a care-kill dichotomy, depending on whether the individual can be cured or is a threat to the fitness of a colony [42,51].

Conceptually, social immunity is analogous to the immune system of a complex multicellular organism (e.g. a Metazoan body) and may even have evolved in response to comparable selective pressures [50,51]. This is because social insect colonies, like a multicellular body, are comprised of two functional components: the queens and males specialized for reproduction and the non-reproducing workers that perform all tasks related to colony maintenance [42]. This division of labour mirrors that of the cells in a body, with the queens/males functioning like germline cells (or gonads) and sterile workers as somatic tissue [39,40,112]. In both cases, neither the germline nor soma elements can survive or replicate without the other, so that when they reproduce, bodies make more bodies, and colonies make more colonies [40,41,50]. For this reason, a social insect colony, although comprising many individuals, can be considered a single reproductive unit that functions like an organism [113]. These organismal qualities emerge from the division of labour between the functional germline and soma, which characterizes both facultative and obligate [112] eusocial insects (the latter of which qualify as "superorganisms", see [39–41,112]). Therefore across these levels of biological organization – organismal bodies and organismal insect colonies – immune systems and social immunity have evolved convergently to mitigate the impact of disease and to maximize fitness, respectively [51].

2.2.2 Evolution of Social Immunity

In recent years, the term social immunity has occasionally been broadened to capture a wider range of behaviours performed by animals living in groups of varying social complexity, including families where all offspring disperse, as well as communally breeding groups [114,115]. Under such *sensu lato* definitions, any behaviour performed by an individual that reduces the disease susceptibility of another may be considered social immunity. Whilst we do not question the importance of these traits and their roles in host-pathogen evolution, we argue that social immunity, as originally defined for eusocial insects [17,51], is a derived trait that evolved when the unit of selection shifted from the individual to the colony, caused by the separation of germline and soma [50]. Such *sensu stricto* social immunity is therefore necessary and essential to protect the entire reproductive entity and maximize its fitness [50]. In eusocial groups, the interests of all individuals are thus sufficiently aligned for cooperation

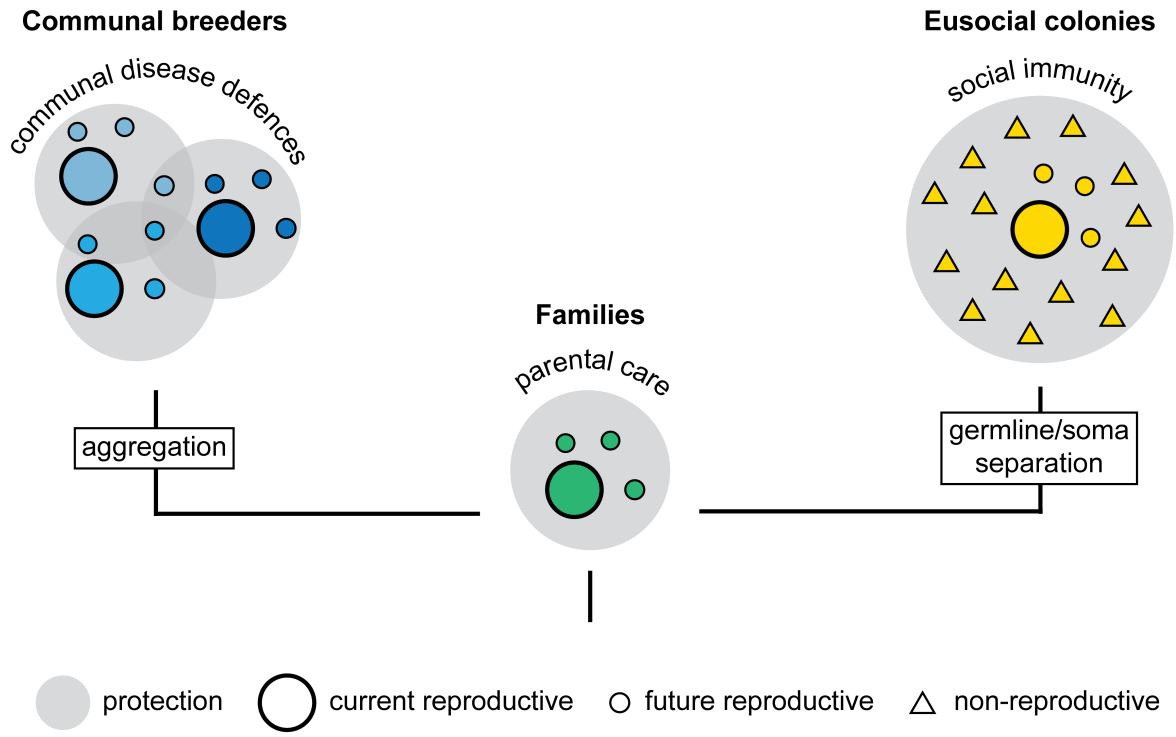

Figure 1. Disease protection in groups. Behaviours that prevent disease in others are present in different forms of social organization. In families with dispersing offspring, parents provide unidirectional disease-protection to their offspring by parental care, thereby enhancing their own fitness. The offspring then disperse instead of helping their parents, and the social group breaks up. In communal breeders, the protection provided by the different group members is additive and a by-product of the aggregation, e.g. disinfection by all group members reducing pathogen load in a communal breeding area [322]. As the potential for conflicts in these aggregations is high, we predict that cheaters should arise and undermine these communal disease defences, by trying to take advantage of the benefits without paying the costs. In eusocial societies, e.g. the colonies of social insects, which evolved from family groups by the separation into reproductive individuals (queens and males; germline) and non-reproductive workers (soma), colony-level protection arises from social immunity. As the level of selection is the reproductive entity of germline and soma, we here see the evolution of altruistic traits that reduce the impact of disease for their kin.

to be high and conflicts low [41,50,113]. Hence, altruistic traits, for example, the expression of sickness signals by infected individuals triggering their own elimination, can evolve via kin selection because they enhance the survival and fitness of the colony. However, the eliminated individual still gains indirect fitness, i.e. by increasing the likelihood that copies of its genes are transferred into the next generation via its kin. Ultimately, social insects workers therefore perform social immunity to maximize their inclusive fitness. In communal breeders [115,116] and family groups, however, conflicts of interest are likely to prevent the emergence of altruistic traits for the benefit of kin, because they would come at a cost to the direct fitness of the individual [113,117] (Fig. 1). Yet, it is exactly these traits that are necessary to make social immunity truly effective and analogous to the evolution of organismal immune systems [50,51].

2.2.3 Emergence of Social Immunity from its Components

Colonies operate without central control and workers have no global picture of what tasks need to be accomplished when or where [118]. Instead, colony organization emerges from the responses of individuals to local cues and dyadic interactions between colony members [118,119]. We suggest that social immunity, like any other complex, concerted task in the colony, is produced through the same means. Yet, despite a wealth of knowledge on the

diversity of individual and collectively expressed defences [17,43,96,97], we have a poor understanding of how they combine to achieve colony-level protection. The "organizational immunity" hypothesis [17,45,95] suggests that the inherent organizational structure of colonies into task-related communities [44,120] and the ability to alter interaction networks in response to pathogens entry should limit intra-colony disease transmission between the members of a social insect colony [121,122]. Despite strong theoretical support, empirical evidence for this hypothesis has so far remained scarce due to the difficulty of studying whole colonies [122,123].

The key to coordinating social immunity responses will likely depend upon the ability of colonies to effectively communicate information about pathogens. Communication about disease might occur directly between insects during one-on-one interactions, e.g. via behavioural changes, or the exchange of regurgitated crop content (trophallaxis), which has recently been found to not only transfer food, but also chemicals used in communication, microRNAs and hormones [124]. In addition to this peer-to-peer information exchange, we propose that the broadcasting of disease information should have evolved to allow for a rapid flow of information through the colony. This is because a host's response needs to be faster than the replication and transmission of the pathogen to prevent a systemic infection. In the body, this is achieved through signalling early in the infection process to generate both local and global immune responses that identify and clear the pathogen [7]. Evidence for similar processes in social insects are limited, but pathogen-exposed termites have been shown to use vibrational cues to trigger a colony-level disease response [125]. However, in social insects chemical communication is typically the main form of communication that is used to coordinate tasks [126] and could play a crucial role in social immunity.

Communication of disease-related information through chemicals should be particularly efficient as volatile signals can be broadcast within the airspace of the nest to reach many individuals at once [127]. Currently, there is no evidence of insects recruiting others to social immunity tasks in a systemic way by releasing volatile chemicals, but several studies have shown that insects exhibit changes in their comparably non-volatile cuticular hydrocarbon profiles upon infection or immune stimulation [56,108,128,129]. These changes are used by nestmates to specifically target the immune-challenged individuals and perform either sanitary care or aggression [128], or elimination behaviour [56,108]. As shown for infected ant brood, these non-volatile signals seem to function in an analogous way to the "find me/eat me" signal that infected cells use to communicate their infection to the immune cells, triggering their elimination [56,130]. Furthermore, cuticular hydrocarbon changes in immune-stimulated honeybee workers have been shown to cause an upregulation of immune genes in their queen [131]. Hence, cuticular chemical cues can act as signals to elicit both social immunity behaviours and the individual immune responses of other colony members. Beyond this, we propose that more systemically-acting volatiles should also have evolved to facilitate the emergence of colony-wide reactions, analogous to the cytokine response of the immune system, sending a systemic signal to all cells in a vertebrate body [7]. Studies investigating how disease information is spread within colonies should help to improve our understanding of the coordination and emergence of social immunity.

2.2.4 Colony-level Avoidance, Resistance and Tolerance to Disease

Emergent colony-level protection arises via different defensive strategies that can be categorized into avoidance, resistance or tolerance strategies. To fight disease, colonies will employ these strategies in combination, but here we will consider them separately to pinpoint to their distinct roles in social immunity.

Avoidance is typically considered as the first line of disease defence in animals [20]. In social insects, individuals can reduce the uptake of pathogens into the colony by avoiding contaminated areas and food [107], closing nest entrances and denying contaminated/infected insects entrance to the colony [132,133]. In general, examples of pathogen avoidance are relatively few in social insects, and recent reports have even found the opposite, i.e. a preference for contaminated nest sites under some circumstances [134,135]. Overall, avoidance seems a relatively unexplored area of disease defence, and more work is needed to understand its relevance in social insects. Yet, the ubiquity of pathogens, among other factors, makes complete avoidance unlikely, meaning that colonies will still need to resist or tolerate diseases [43].

Resistance combines all responses that reduce pathogen load and can be achieved by decreasing the probability of infection or by lowering the amount of/clearing pathogens after infection [30]. At the colony-level, resistance is achieved by sanitizing the nest with antimicrobials and removing dead individuals to reduce the probability of microbial growth and the potential for infection [136–143]. In addition to general nest hygiene, targeted sanitary care of contaminated insects, such as cleaning of the body surface by allogrooming and prohibiting pathogen germination or growth by disinfection, reduces their risk of infection [55,106,107,144–147] . These measures are thought to be more important for ants and termites, which nest in direct contact with soil and wood containing a large number and diversity of generalist pathogens, e.g. fungal spores [48] (see 3.1). If hygiene and sanitary care fail and colony members become infected, the colony can reduce pathogen load by preventing infections spreading to uninfected colony members. To that end, infected brood is removed or destroyed and adults aggressively excluded [56,105,108,148,149]. Contaminated workers also leave the brood chamber [52], and moribund individuals leave the colony independent of their infection state, which could decrease their interaction rates with susceptible nestmates [110,150,151]. Interestingly, infected honeybees switch faster to out-of-hive tasks, which could reduce disease transmission within the hive [152]. Indeed, an experimental increase in colony pace, achieved through a faster worker turnover, reduced pathogen load in the colony [153]. Hence, social insects have evolved many ways to reduce the load and prevent infections of a wide diversity of pathogens.

Tolerance is the capacity of a host to limit the negative impact of an infection on its fitness without directly affecting pathogen load itself [30,154,155]. Although the underlying mechanisms of tolerance are often unclear, they revolve around reducing the damage that arises either directly from the pathogen or indirectly through an immune response aimed at the pathogen [30,155,156]. Tolerance has so far received less attention than resistance in studies of animal disease defence, but is a growing area of research [155]. We predict that tolerance may play an important role in social immunity, as colony-level tolerance mechanisms would allow colonies to cope with worker losses due to infection [150,151] or the elimination of infected workers by nestmates[56,105,111]. Damage to the worker force could also arise through collateral damage if healthy workers are erroneously killed or damaged by social immunity behaviours, similar to immunopathology in a body [156]. Evidence of ‘social immunopathology’ is lacking but could exist because antimicrobials used may be toxic to the insects themselves (e.g. cytotoxic formic acid [55,157]) and mechanisms to eliminate infected colony members may not be perfect [56,149].

Although colony-level tolerance mechanisms are unknown, we predict that they should involve the efficient replacement of lost workers with new ones to avoid a decrease in factors

affecting fitness, such as colony productivity. Typically, social insect colonies exhibit temporal polyethism [44,93,120], where the youngest workers perform in-nest tasks (e.g. nursing the brood), but progress to more dangerous out-of-nest tasks as they age (e.g. guarding and foraging). Like different organs in a body [30], we postulate that these different groups of workers should have distinct intrinsic tolerance capacities. For example, the eldest workers, the foragers, can be replaced easily by younger workers switching to foraging tasks [44,93,158], whilst the youngest workers, the nurses, are regenerated from brood, which takes longer than forager substitution [93]. Moreover, because the nurses raise their own replacements, a loss of these workers will severely limit the colony's ability to regenerate its worker force. Thus, it would be interesting to examine whether 'tissue-specific tolerance' [30] exists between the different worker groups in social insect colonies.

Tolerance mechanisms can function after the damage occurred, such as a transient increase in worker production following an infection [159]. However, replacement individuals may already be produced prophylactically. In some species, as many as 45% of the worker force appear to specialize in inactivity [160], and could act as a buffer for 'soma damage'. Moreover, tolerance mechanisms may not only ameliorate damage arising from the complete loss of workers, but already from reductions in functionality. For example, the negative effects of parasitized workers that no longer contribute to colony productivity appear to be tolerated by the colony through compensatory action of healthy workers [161].

Unlike the workers, the queen is often irreplaceable and her loss cannot be tolerated. Like irreplaceable cells in a body, such as the brain and the gonads, queens are therefore thought to receive special immune protection, known as an immune privilege [17,30,51]. Hence, tolerance may not always be possible and, generally, it is expected to function up to a certain point, beyond which the damage caused by disease will no longer be tolerated. Resistance mechanisms should then come into play to actively reduce pathogen load [30].

Avoidance, resistance and tolerance will act together to defend the colony, and the relative investment into each strategy will depend on a number of factors [30]. For example, to tolerate the loss of workers, a colony needs to be sufficiently large and/or able to produce new workers fast. Hence, species with small colonies and young ones investing into growth are expected to tolerate worker losses poorly, so they might invest relatively more into resistance mechanisms, if the costs of resistance do not cause trade-offs with reproduction. Investment into resistance versus tolerance will also depend on pathogen infection mode and virulence, i.e. the harm it causes in the host upon successful infection. As examples, orally infecting viruses of low virulence may be tolerated by producing more workers to buffer the reduced activity of infected workers and occasional deaths, whilst obligately-killing fungi that kill their hosts should select for resistance [43]. Studies on the role of tolerance in social immunity and its interaction with other defence strategies could prove to be an exciting avenue for future research.

2.2.5 Fitness Effects of Social Immunity

To understand how social immunity is selected for and evolves, we need to study its long-term consequences on colony fitness. Ultimately, social insect fitness is measured as the number of new daughter colonies a parental colony contributes to the next generation [70,162]. Ideally, to understand the evolutionary importance of social immunity, fitness would be compared between colonies expressing and lacking social immunity. Performing such experiments over several generations, whilst excluding potentially confounding effects, is challenging in most cases, as many species rely on poorly understood environmental

factors to trigger mating, so that only parts of their lifecycle can be observed in the laboratory.

Nonetheless, fitness estimates, such as the number of reproductive offspring (daughter queens and males) produced per colony can provide good, short-term proxies for colony fitness. These estimates can be obtained from field [163] and laboratory colonies, by counting their numbers and determining classical life-history parameters, such as body size or immune function [164–166]. The latter is particularly important when making predictions about their likelihood of surviving the non-reproductive (ergonomic) colony-founding phase, only after which they reproduce and gain fitness themselves [70].

To increase the chances of a colony gaining fitness, founding queens and longer-lived males (e.g. termite kings) may be immune-primed by their maternal colony to enhance their survival upon a later pathogen challenge [167]. So far, only maternal transgenerational immune priming (TGIP) from the mother queens to their offspring has been described in social insects [168,169], but TGIP can also occur via the father in other insects [170]. However, as the parents' role in social insect colonies ends with mating (males) and egg laying (queens) in mature colonies, TGIP may be more likely to occur via the nurses that actually raise and care for the brood [171]. Worker-mediated TGIP may be particularly important and necessary in social insects as the queens are exceptionally long-lived and mate once at the beginning of their lives [172] and there is a protracted delay between egg laying and the emergence of adults. Hence, the immunological experience of the parents may be out of sync with the current pathogen pressure facing emerging offspring, and only reflects a small fraction of the pathogen diversity. However, the combined immunological experience of the workerforce is more likely to reflect the prevailing pathogen community. Thus, we propose that studies are needed to test for the existence and importance of tripartite – maternal, paternal, sibling – TGIP in social insects and its impact on fitness.

2.3 Eco-evolutionary dynamics of social insect host-pathogen interactions

In social insects, pathogens can spread both within and between colonies [42]. However, because social insects exist as populations of colonies [42,70], true horizontal disease transmission is defined as transmission between colonies of the same population and generation [42]. It can occur directly between insects from different colonies, as well as indirectly through environmental transmission. Vertical transmission in social insects, on the other hand, is any form of disease transmission from a parent colony to its offspring colonies [42,43]. Understanding how pathogens are transmitted and interact with their social insect hosts in the population is important, as these factors will affect the selection pressures producing and maintaining social immunity. Importantly, social insect lineages are expected to vary their host-pathogen interactions due to substantial differences in their life histories. Here, we highlight the major life-history parameters of the different social insects and the effects they are expected to have on host-pathogen interactions (but see [42,43] for a comprehensive overview). We then explore how this may select for social immunity, as well as how social immunity in turn influences the evolution of pathogens.

2.3.1 Host Life Histories and Pathogen Exposure, Transmission and Virulence

Host life history will influence where and how pathogens are encountered and recruited. For instance, ants and termites are both highly territorial, reducing the number of opportunities pathogens have for direct horizontal transmission. This means that the majority of their

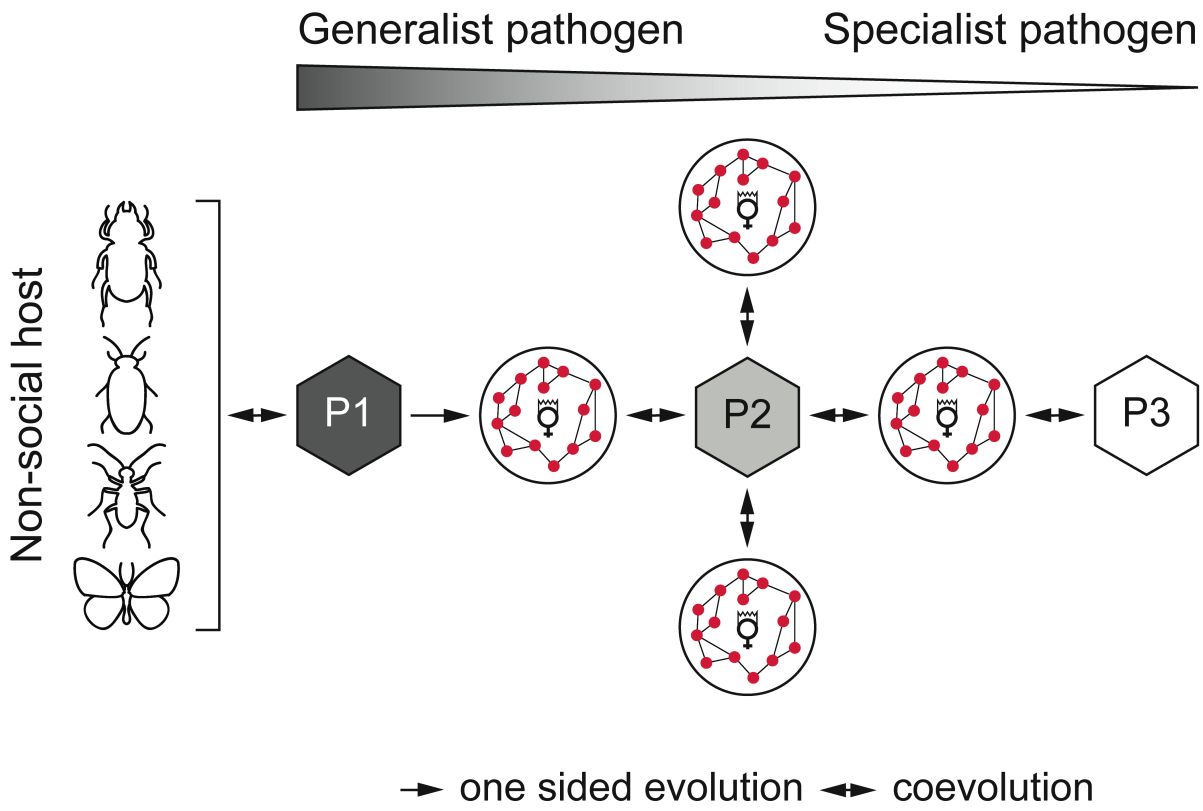

Figure 2) Host-pathogen evolution. Pathogens can interact with hosts at different degrees of host range, from the capacity to successfully infect many hosts (generalism) to a very limited host range (increasing specialism). The degree of host specificity defines their evolutionary dynamics with their hosts [323]. Generalist pathogens like **P1** can coevolve with multiple non-social hosts, thereby reaching high abundance, which exerts a strong selective pressure on social insect hosts. This has led to the evolution of highly effective social immunity against such generalists, making it unlikely for the pathogen to complete its lifecycle when infecting a social insect host (dead-end for the pathogen), limiting the likelihood that it evolves counter-adaptations, favoring one-sided evolution of the host. Pathogens of intermediate degree of specialism like **P2** can infect multiple social hosts, with which they may diffusely coevolve (non-reciprocally [324]). Specialist pathogens like **P3** that only infect a single social host species coevolve reciprocally with their social host.

pathogens will be encountered and acquired from the environment [43,173], via durable long-lasting stages that "sit and wait" until they are encountered by a host [42,174]. These pathogens are typically fungi that can actively penetrate the host cuticle or spore-forming bacteria that cause infections after ingestion (*per os*). Environmentally transmitted pathogens encounter new hosts stochastically, so typically evolve broader host ranges (generalism) to increase their chances of reproduction [87]. Hence, many of the pathogens ant and termites experience will be generalists (Fig. 2). Several selection pressures will thus affect pathogen virulence, and the overall outcome cannot easily be generalized [43]. On one hand, pathogen virulence typically decreases with infection of multiple host species, [175], (but see [176]), whilst environmental transmission selects for increased pathogen virulence [42,174].

In contrast, bees and wasps are expected to encounter and recruit more pathogens *per os* that are transmitted directly between insects of different colonies [43], e.g. when they drift from one colony and enter another [177–179]. In addition, indirect horizontal transmission is facilitated by the use of shared food resources, such as flowers [43]. These transient food patches are visited by insects of multiple colonies and species, so that they may act like 'disease hubs' promoting the transmission of pathogens between them [43]. Transmission

will mostly occur via the faecal-oral route [180,181]: infected insects contaminate resources, e.g. flowers, leaving behind infectious propagules that can infect susceptible insects using the same resource in the near future. The stable, frequent transmission of pathogens between colonies of the same or closely related species, e.g. within Apidae and Vespidae, will favour more specialist pathogens in bees and wasps than in either ants or termites [42,43]. Pathogen virulence is expected to increase under frequent horizontal transmission [182,183], yet, high virulence evolution is expected to be counteracted by the necessity of infected bees and wasps to still be able to forage to disseminate the pathogen.

Ants and wasps will also encounter pathogens from their food as they hunt or scavenge on other insect and arthropod species [43]. In addition, ants tend honeydew-producing insects, such as aphids, as a source of carbohydrates. These intimate interactions with other species may lead to disease spill over events. However, infection likelihood and virulence are hard to predict. For example, a generalist virus acquired from feeding on an infected corpse may cause virulent infections. However, ants that encounter obligate aphid pathogens (e.g. *Pandora neoaphidis*) whilst collecting honeydew are unlikely to contract the disease themselves [184].

Vertical transmission may play a key role in the spread and persistence of pathogens in social insect populations. In social insects, vertical transmission includes both *sensu strictu* vertical transmission, where a pathogen is passed from a queen directly to a daughter queen, but also any type of transmission that leads to the infection of daughter colonies. For example, this could be via the workers that accompany the queens of some species during colony foundation. Typically though, most social insect queens found colonies alone, or, as mated pairs in termites. The differences between these types of colony foundation may affect the evolution of virulence in vertically transmitting pathogens. When queens found colonies alone, selection should favour pathogens with relatively benign effects, as the queens need to survive and reproduce before the pathogen can disperse again through daughter queens [42,43]. However, this constraint may be relaxed in species where workers from the parental colony leave with the daughter queens, because the workers may buffer the negative impacts of virulent pathogens. However, pathogen virulence should still depend on the relative frequency of horizontal to vertical transmission events [185].

As previously pointed out [42,43], virulence predictions for social insect pathogens are hard to make as they have diverse and complex host interactions – virulence can conceivably evolve in any direction [42,43] and may even be context dependent [186]. However, overall differences between host-pathogen associations across the social insects are clear and will cause a skew in the types of pathogens the different social insect lineages encounter [42,43]. Namely, more generalists in ants and termites and more specialists in bees and wasps [43]. This skew is also present in established model systems for the study of social immunity, and therefore likely reflects natural host-pathogen associations. To gain a fuller understanding of social host–pathogen evolution, we nonetheless encourage future studies investigating the full range of pathogens social insects encounter, as well as how pathogens persist in social insect populations that appear to defy the above association bias (e.g. viral infections in ants and termites).

2.3.2 Social Immunity and Host–Pathogen Evolution

How pathogens select for social immunity and, in turn, how social immunity influences pathogen evolution, remain open questions that require further study [8]. However, between-colony variation in the expression of social immunity behaviours, such as e.g. grooming

Sidebar 1: Generalist fungal pathogens in ants

Recently, the ecological relevance of generalist fungi in social immunity research has been questioned [325]. However, generalist fungal pathogens have been infecting ants for millions of years [326], and to date, the cosmopolitan *Metarhizium* and *Beauveria* are the best-studied species [85,327]. These obligately-killing pathogens produce very high numbers of infectious propagules (approx. 12 million per infectious cadaver), which reach high abundances in the environment, either freely in the soil (up to 5000 infectious conidiospores/g soil) [46–48,89,202], or in association with plants [328]. Once in contact with insect cuticle, they attach and actively penetrate into their host, where they can cause lethal infections [329,330]. In the field, deadly infections are estimated to be up to 10% of ant workers and 1% of colony-founding queens that have yet to start digging into the soil. These reports are likely underestimates, however, as cadavers are inconspicuous in the field (e.g. lost in leaf litter or kept in colony graveyards [266]) and can be destroyed by ants to prevent sporulation [56]. As these fungi can be reared in the laboratory, they are excellent model systems to study social immunity against generalists. Most studies use topical applications of between 2-20% of the conidiospores released by a single cadaver, to reach a lethal dose (LD) 50 in the laboratory [53,55,89,105,149]. Yet, since many conidiospores immediately fall off during this mode of application, the actual infective LD50 dose is much less, and far below 1% of a cadaver-load of conidiospores; indeed deadly infection can also occur at very low conidiospore numbers (<10 per insect), albeit with a lower probability (M. Konrad, A.V. Grasse, SC, unpubl.). Studying these generalists under controlled laboratory conditions allows for detailed experiments investigating the evolution of collective host defenses against the constant selection pressure these pathogens impose on social insects.

[150], exists, which selection can, in theory, act upon. Genetic differences between patrilines can increase the diversity of defences expressed within colonies [187], and social immunity traits like hygienic removal of brood in honeybees has been shown to be heritable and can be artificially selected for, with direct fitness consequences to colony-level disease protection [111]. Hence, pathogen-induced selection, acting on standing variation in social insect populations, is therefore likely maintaining and driving the evolution of social immunity.

The evolutionary outcomes of host-pathogen interactions will depend on many factors, such as the type of pathogen and its host specificity. For example, generalist entomopathogenic fungi with broad host ranges release large quantities of infectious conidiospores from corpses, to maximize their chances of infecting a new host in the future [87] (see sidebar 1). The high abundance of these spores in the environment should place a strong selection pressure on ant and termite colonies to evolve social immunity traits that reduce their impact. However, this evolutionary process is asymmetric: one-sided adaptations may evolve in the host to combat the high pathogen burden, whilst the pathogen itself does not evolve any reciprocal specialist traits, because it switches hosts across generations. Moreover, social immunity defences in both ants and termites can prevent generalist pathogens from replicating within the colony, meaning that such infections are likely evolutionary 'dead ends' (Fig. 2).

Interestingly, however, several specialist fungi that cause endemic infections in ants are able to persistently infect the same colony across years (see sidebar 2). They do this by manipulating their hosts into leaving the nest before becoming infectious, so that they avoid the social immunity defences that prevent reproduction in generalist pathogens. Whether host manipulation evolved specifically to overcome social immunity defences is unknown. However, in response, ant colonies have evolved a reciprocal behaviour – they regularly search around the colony and attempt to remove infected corpses before the pathogen becomes infectious. This is therefore suggestive of a classical evolutionary arms race between a pathogen able to overcome social immunity and the evolution of a new, antagonistic social immunity defence in response. Such reciprocal coevolution occurs when pathogens are specialized to infect to a single host species. In addition, non-reciprocal

coevolution can occur through diffuse interactions between multiple linages [188], for example, between multiple species of bees and wasps that exchange pathogens on flowers (Fig. 2).

Social insect colonies are thus exposed to a wide diversity of pathogens, which can usually infect a few to potentially many hosts. Hence, evolutionary interactions between social insects and their pathogens are likely to be complex. For example co-infections of multiple pathogens seem to be the rule rather than the exception [189]. How co-infections of colonies influence the evolution of social immunity is an interesting question. Generally, behavioural responses are often effective against a broad range of pathogens. As an example, grooming and chemical disinfections may reduce infection probability of most pathogens that infect via the cuticle, relatively independent of the exact pathogen strain or species [103,190,191]. Ants also show increased grooming levels with pathogen experience [106,146,192], and we suggest this should also cross-protectively act against other, comparable pathogens entering the colony. Hence, the evolution of social immunity may play an important role in fighting multiple pathogens present in a colony.

Sidebar 2: Specialist fungal pathogens in ants

Several specialist fungal pathogens of ants exist [331,332], which fall into two categories: pathogens of very low virulence, which may not place strong selection pressure on their hosts, and highly virulent pathogens that seem to have evolved adaptations to evade social immunity. Species of the order Laboulbeniales (such as *Rickia* and *Laboulbenia*) anchor their large thalli into the cuticle of living ants and have low virulence [333], potentially even being beneficial for their hosts by providing protection against generalist pathogens [54]. In contrast, *Pandora* [223] and *Ophiocordyceps* spp. [331] are obligate killers. In parallel, they have evolved the ability to manipulate their hosts into leaving the nest and climbing nearby plant stems, where the insects attach, die and become infectious [223,334].Conspicuous fruiting bodies then emerge and release relatively few, large infectious ascospores that infect foraging workers [104]. Their conspicuous cadavers make them an excellent field study system, as the number of corpses around ant nests can easily be determined [104,335], and was found to be relatively few per colony [104,332]. Thus far, infections in the laboratory have only been established via non-natural route of injection of hyphae [336,337]. It would be therefore be highly valuable to establish controlled laboratory infections via the natural infection route, e.g. using topical applications of cuticle-attaching and penetrating ascospores – as in the generalist fungi (sidebar 1) – to study all aspects of their biology. Specialist fungi are interesting models to study co-evolution between social insects and their pathogen, and in particular, to understand how specialist pathogens can evolve to evade social immunity.

2.4 Conclusions and future outlook

With a well-established conceptual framework, an increasing knowledge of social host-pathogen interactions, and novel technological developments [17,42,43,51,122], we feel it is timely and feasible to experimentally address how social immunity emerges from its mechanistic components. That is, how do different social immunity actions, based on dyadic interactions and local cues, scale up, interact and synergize to reach colony-level protection? For this, we need to take whole colony perspectives, which analyse how the organization of social insect colonies contributes to social immunity [17]. Novel technologies like automated monitoring, machine learning and network analyses [44,193–195] offer us powerful tools to address both the organizational immunity hypothesis, as well as a refined view of how local interactions and information exchange can produce emergent properties [44,196]. Combining these technologies with methods to quantify pathogen spread [53,153] offers a potentially promising approach to understand how disease defences functions at the colony-level [122].

To understand how social immunity evolves and, in turn, affects pathogen evolution, we require long-term studies measuring the fitness of both parties. Whilst challenging, this can

be approached using both field and laboratory studies. Longitudinal field studies over multiple years (across time) [162], or local adaptation approaches (across space) could be used to address if and how social immunity responses adapt and evolve to specific pathogen communities. In the laboratory, evolution experiments, including co- and one sided-evolution, can shed light on evolutionary interactions that are hard to study in the field. For example, serial infections of generalist pathogens with the same social insect host could be used to investigate how specialist pathogens evolve adaptations to overcome social immunity defences. Indeed, with on-going biodiversity losses, caused by, for example, invasive social insects [197,198], changes in the prevailing host community are likely to disrupt established host-pathogen dynamics.

To gain a fuller insight into the complexity of host-pathogen interactions in social insects, deep sequencing techniques provide potentially powerful tools to tackle current gaps in our knowledge. Furthermore, comparative genome analyses will allow us to determine how, for example, the evolution of the immune system is affected by social immunity. To conclude, we believe that developing a single socio-eco-evo-immunological framework for social immunity will develop our understanding of its role in social evolution and host-microbe interactions.

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3. Destructive disinfection of infected brood prevents systemic disease spread in ant colonies

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Abstract
Social insects protect their colonies from infectious disease through collective social immunity defences. In ants, workers first try to prevent infection of colony members. Here, we show that if this fails and a pathogen (*Metarhizium brunneum*) establishes an infection, ants (*Lasius neglectus*) employ an efficient multicomponent behaviour, termed destructive disinfection, to prevent further spread of disease through the colony. Ants specifically target infected pupae during the pathogen's non-contagious incubation period, utilising chemical "sickness cues" emitted by pupae. They then remove the pupal cocoon, perforate its cuticle and administer antimicrobial poison, which enters the body and prevents pathogen replication from the inside out. Like the immune system of a body that specifically targets and eliminates infected cells, ants destroy infected brood to stop the pathogen from completing its lifecycle, thus protecting the rest of the colony. Hence, the same principles of disease defence apply at different levels of biological organisation.

Keywords
superorganism; sickness cues; collective behaviour; fungal infection

Author contributions
Conceived and designed study: CDP, ST, MB, SC
Performed experiments: CDP
Chemical analysis and peak identification: LVU, FW, TS
Analysed data: CDP
Wrote paper: CDP, SC

Experimental work in detail
Destructive disinfection characterisation (Figure 1): CDP (100%)
Cuticular chemical assay and analysis (Figure 2): CDP (30%), LVU, FW, TS
Effect of destructive disinfection (Figure 3): CDP (100%)
Disease transmission from corpses (Figure 4): CDP (100%)

3.1 Introduction

Pathogen replication and transmission from infectious to susceptible hosts is key to the success of contagious diseases [7]. Social animals are therefore expected to experience a greater risk of disease outbreaks than solitary species, because their higher number of within-group interactions will promote pathogen spread [1,8,199]. As a consequence, traits that mitigate this cost should have been selected for in group-living animals as an essential adaptation to social life [3,5].

Eusocial insects (termites, ants and the social bees and wasps) live in complex societies that are ecologically successful and diverse. They are typically single-family colonies comprising one or a few reproducing queens and many sterile workers. Both of these castes are highly interdependent: the queens are morphologically specialised for reproduction and cannot survive without the assistance of the workers; conversely, the workers cannot reproduce, but gain inclusive fitness by raising the queen's offspring [91]. Consequently, social insects societies have become single reproductive units, where natural selection acts on the colony instead of its individual members [32,50]. This has parallels to the evolution of complex multicellular organisms, where sterile somatic tissue and germ line cells form a single reproducing body. Hence, social insect colonies are often termed "superorganisms" and their emergence is considered a major evolutionary transition [32,39,40,50]. Since evolution favours the survival of the colony over its members, selection has resulted in a plethora of cooperative and altruistic traits that workers perform to protect the colony from harm [3,17,50,200]. In particular, social insects have evolved physiological and behavioural adaptations that limit the colony-level impact of infectious diseases, which could otherwise spread easily due to the intimate social interactions between colony members [17,97,115,122]. These defences are performed collectively by the workers and form a layer of protection known as social immunity that, like the immune system of a body, protects the colony from invading pathogens [17,51].

Our understanding of how social immunity functions is based mostly on the first line of defence that reduces the probability of pathogen exposure and infection. It is well known for example that social insects avoid pathogens, like fungal spores, in their environment, and perform sanitary care when nestmates come into contact with them [55,107,144,146,201]. In ants, sanitary care involves grooming and the use of antimicrobial secretions to mechanically remove and chemically disinfect the pathogen, reducing the likelihood that pathogen exposure leads to the development of an infection [55,144]. However, it remains poorly understood what happens when sanitary care fails and a pathogen successfully infects an ant, with the consequent potential to create an epidemic. In a body, infected cells are eliminated by the immune system to prevent the proliferation and systemic spread of pathogens through the tissue. Since infected ants become highly contagious to their nestmates [202,203], we hypothesised that they should have evolved an analogous mechanism to detect and contain lethal infections in individuals as early as possible, to prevent disease outbreaks in the colony.

To test this hypothesis, we exposed pupae of the invasive garden ant, *Lasius neglectus,* to a generalist fungal pathogen, *Metarhizium brunneum*. When the infectious conidiospores of this fungus come into contact with insect cuticle, they attach, germinate and penetrate the host cuticle within 48 h to cause internal infections. After a short, non-infectious incubation period of a few days, a successful fungal infection then induces host death, after which the fungus replicates and releases millions of new infectious conidiospores in a process called sporulation [84,202]. Previous work found that brood infected with *Metarhizium* is removed

from the brood chamber, however, it is unknown how the ants then respond to the infection [105,149]. Here we demonstrate that ants detect infected pupae during the pathogen's non-infectious incubation period and react by performing a multicomponent behaviour. To investigate this response we used a series of behavioural and chemical experiments to determine its function and underlying mechanisms. Finally, we tested the impact of the multicomponent behaviour on the pathogen's ability to complete its lifecycle and cause a systemic colony infection.

3.2 Results

3.2.1 Destructive disinfection of lethally infected pupae

We exposed ant pupae to either one of three dosages of *Metarhizium* conidiospores or a sham control. We observed that ants tending pathogen-exposed pupae prematurely removed the pupae from their cocoons in a behaviour we termed "unpacking", whereas control pupae were left cocooned (Figure 1A-B, Video 1; Cox proportional hazards regression: likelihood ratio test (LR) $\chi^2 = 55.48$, df = 3, $P = 0.001$; post hoc comparisons: control vs. low, $P = 0.004$; low vs. medium, $P = 0.006$; medium vs. high = 0.024; all others, $P = 0.001$). Unpacking occurred between 2-10 d after pathogen exposure, but sooner and more frequently at higher conidiospore dosages (Figure 1B). As unpacking was a belated response to pathogen exposure and we were unable to remove any conidiospores from the cocoon or the unpacked pupae (Figure 1 – Figure supplement 1), we concluded that the ants were not performing unpacking to simply dispose of the contaminated cocoons. Instead, we postulated that unpacking was a response to successful infection. At the time of unpacking, the majority of pupae were still alive (Figure 1 – Figure supplement 2) and fungal outgrowth had not yet occurred (Figure 1F). Hence, to test if the ants were reacting to early-stage infections, we removed both unpacked and non-unpacked pathogen-exposed cocooned pupae from the ants and incubated them under optimal conditions for fungal outgrowth. We found that, on average across the conidiospore dosages, 85% of unpacked pupae harboured infections that sporulated in the absence of the ants. In contrast, only 25% of non-unpacked pupae were infected (Figure 1 – Figure supplement 3; generalised linear model [GLM]: overall LR χ^2 = 21.52, df = 3, $P = 0.001$; cocooned vs. unpacked pupae: LR $\chi^2 = 18.5$, df = 1, $P = 0.001$; conidiospore dose: LR $\chi^2 = 0.42$, df = 2, $P = 0.81$). We therefore concluded that the ants were detecting and unpacking pupae with lethal infections during the asymptomatic incubation period of the pathogen's lifecycle. At this time point the fungus is non-infectious and so there is no risk of the ants contracting the disease.

Next, we filmed ants presented with pathogen-exposed pupae and compared their behaviour before and after unpacking. Prior to unpacking, we observed the typical sanitary care behaviours reported in previous studies [55,145,146,202]. Namely, the ants groomed the pupae (Figure 1C), which has the dual function of removing the conidiospores and applying the ants' antimicrobial poison [55]. In *L. neglectus*, the poison is mostly formic acid and is emitted from the acidopore at the abdominal tip, where the ants actively suck it up and transiently store it in their mouths until application during grooming. Additionally, the ants can spray their poison directly from the acidopore; yet, this behaviour is rarely expressed during sanitary care (about once every 28 h; Figure 1D) [55]. However, after unpacking, we observed a set of behaviours markedly different to sanitary care (Figure 1A, Video 1). The ants sprayed the pupae with poison from their acidopore approx. 15-times more frequently than during sanitary care (~ 13-times/d; Figure 1D; generalised linear mixed model [GLMM]: LR $\chi^2 = 17.04$, df = 1, $P = 0.001$), and increased grooming by 50% (Figure 1C; linear mixed

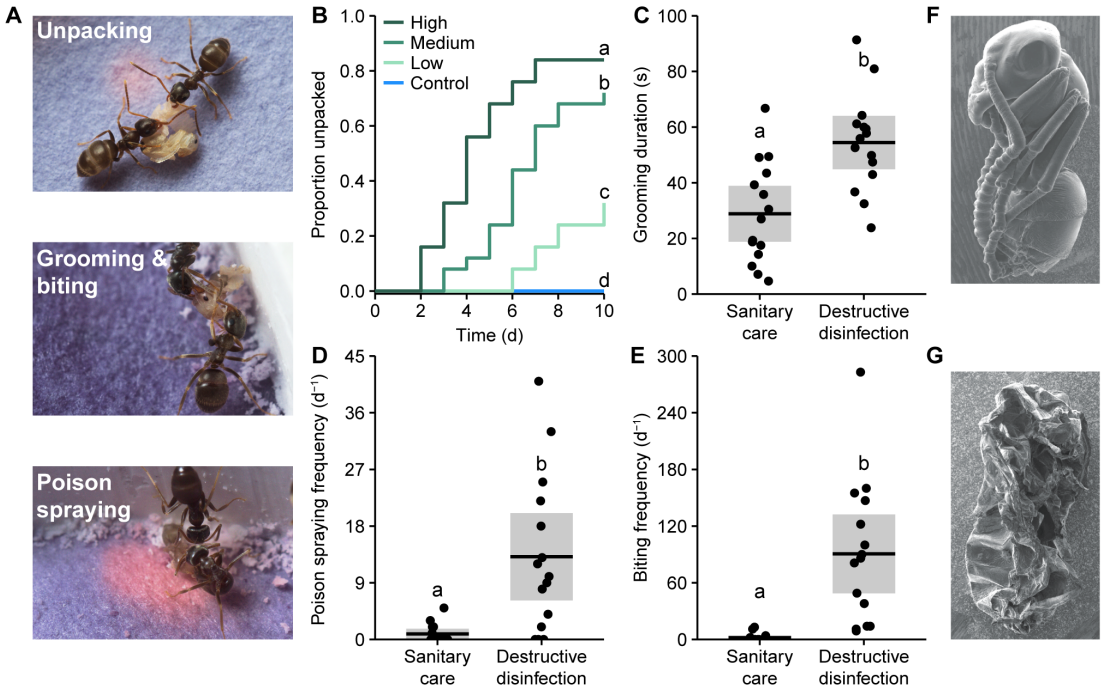

Figure 1. Ants perform destructive disinfection in response to lethal fungal infections of pupae. (A) Destructive disinfection starts with the unpacking of pupae from their cocoons and is followed by grooming, biting and poison spraying (ants housed on blue pH-sensitive paper to visualise acidic poison spraying, which shows up pink). (B) Unpacking occurred when pupae were exposed to fungal conidiospores and was dose-dependent, happening sooner and in higher amounts as the dose of conidiospores increased (letters denote groups that differ significantly in post hoc comparisons [model revelling; $P < 0.05$]). (C-E) Comparison of the ants' behaviour between sanitary care and destructive disinfection. Destructive disinfection is characterised by increases in grooming duration, poison spraying frequency and biting frequency (all data points displayed; lines ± shaded boxes show mean ± 95% confidence intervals [CI]; letters denote groups that differ significantly in logistic regressions [$P < 0.05$]). (F) Scanning electron micrographs (SEM) of an asymptomatic infected pupa immediately after unpacking, and (G) of a destructively disinfected pupa 24 h later.

effects regression [LMER]: LR $\chi^2 = 145.26$, df = 1, $P = 0.001$). Given that there was no fungus to remove at the time of unpacking, the increase in grooming probably functioned solely to apply poison from the oral store [55]. Furthermore, the ants repeatedly bit the pupae to make perforations in their cuticles (Figure 1E; GLMM: LR $\chi^2 = 39.44$, df = 1, $P = 0.001$). Together these three behaviours resulted in the death of the pupae and left their corpses heavily damaged and coated in the ants' poison (Figure 1G, Figure 1 – Figure supplements 2 and 4). Accordingly, we named the combination of unpacking, grooming, poison spraying and biting "destructive disinfection", and performed a series of experiments to determine its function.

3.2.2 Chemical detection of internal infections

Firstly, we wanted to know how the ants identify internal infections during the pathogen's non-contagious incubation period, when pupae were still alive and showed no external signs of disease. As ants use chemical compounds on their cuticles to communicate complex physiological information to nestmates [126], we speculated that infected pupae may produce olfactory sickness cues. We washed infected pupae in pentane solvent to reduce the abundance of their cuticular hydrocarbons (CHCs). When pentane-washed pupae were presented to ants, there was a 72% reduction in unpacking compared to both non- and water-washed infected pupae (Figure 2A; GLM: LR $\chi^2 = 12.2$, df = 2, $P = 0.002$; Tukey post hoc

comparisons: water-washed vs. non-washed, $P = 0.79$; all others, $P = 0.009$). As pentane-washed pupae had lower abundances of CHCs (Figure 2 – Figure supplement 1), this result indicates that the ants use one or more cuticle compounds to detect the infections.

Gas chromatography-mass spectrometry (GC–MS) analysis of the solvent wash confirmed that unpacked pupae have distinct chemical profiles compared to non-infected control pupae, whilst cocooned (non-unpacked) pathogen-exposed pupae were intermediate (Figure 2B, Figure 2 – Figure supplement 2; perMANOVA: $F = 1.49$, df = 46, $P = 0.002$; post hoc

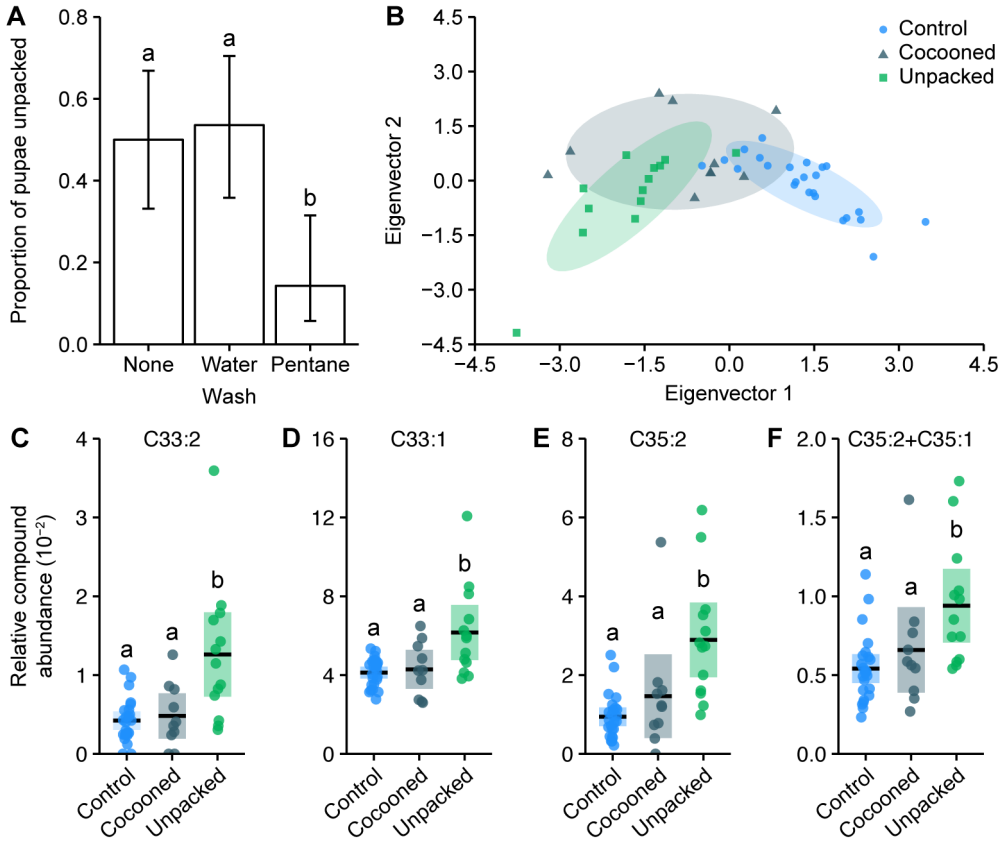

Figure 2. Destructive disinfection is induced by changes in the chemical profile of infected pupae.
(A) Pupae washed in pentane solvent to reduce the abundance of their cuticular hydrocarbons (CHCs) were unpacked less than unwashed or water-washed pupae (positive and handling controls, respectively; error bars show ± 95% CI; letters specify significant Tukey post hoc comparisons [$P < 0.05$]). (B) Unpacked pathogen-exposed pupae have distinct chemical profiles compared to sham-treated control pupae. Pathogen-exposed pupae that were not unpacked (cocooned group) have intermediate profiles (axes show discriminant analysis of principle components eigenvectors). (C-F) The four CHCs with higher relative abundances on unpacked pupae compared to both control and cocooned pupae, (C) Tritriacontadiene, C33:2 (D), Tritriacontene, C33:1 (E), Pentatriacontadiene, C35:2 (F) co-eluting Pentatriacontadiene and Pentatriacontene, C35:2+C35:1 (all data points displayed; line ± shaded box show mean ± 95% CI; letters specify groups that differ significantly in KW test post hoc comparisons [$P < 0.05$]).

perMANOVA comparisons: unpacked vs. control, $P = 0.003$; unpacked vs. cocooned, $P = 0.79$; cocooned vs. control, $P = 0.08$). Most chemical messages in social insects are encoded by quantitative shifts of several compounds [126]. Correspondingly, we found that 8 out of the 24 CHCs identified (Table 1) had higher relative abundances on unpacked pupae compared to control pupae (Figure 2C-F, Figure 2 – Figure supplement 2; all Kruskal-Wallis [KW] test

statistics and post hoc comparisons in Table 2). Moreover, four of these CHCs were also present in relatively higher quantities on unpacked pupae compared to the non-unpacked cocooned pupae. Hence, several specific CHCs probably accumulate on infected pupae over time, eventually reaching an amount that, relative to the other compounds, is sufficient to elicit destructive disinfection. This corresponds to current models of social insect behaviour, where the likelihood of a response depends on stimuli exceeding a certain threshold [204,205]. Interestingly, the four CHCs specifically increased on unpacked pupae were all long-chained CHCs (carbon chain length C_{33-35}) with a low volatility, meaning that the ants have to be close to or touching the pupae to detect them [206]. As ants keep pupae in large piles, using low-volatility CHCs may be important so that the ants accurately identify the sick pupae and do not mistakenly destroy healthy ones.

3.2.3 Destructive disinfection prevents pathogen replication

We next tested if destructive disinfection prevents pupal infections from replicating and becoming infectious. Pathogen-exposed pupae were kept with groups of ants (8 ants per pupae per group) until unpacking. They were then left with the ants for a further 1 or 5 d before being removed and incubated for fungal growth. We compared the number that subsequently sporulated to pathogen-exposed pupae kept without ants. Whilst 88% of pupae contracted infections, destructive disinfection significantly reduced the proportion of pupae that sporulated and hence became infectious (Figure 3A; GLM: LR $\chi^2 = 40.47$, df = 2, $P = 0.001$; Tukey post hoc comparisons: 1 vs. 5 d, $P = 0.04$; all others, $P = 0.001$). After only 1 d, the number of destructively disinfected pupae that sporulated decreased by 65%. With more time, the ants could reduce the number of pupae sporulating even further by 95%. Since the pupae were removed from the ants for fungal incubation, we can conclude that destructive disinfection permanently prevents pathogen replication. We repeated this experiment with a smaller number of ants (3 ants per pupae per group) to investigate how group size influences the success of destructive disinfection. Smaller groups of ants were less efficient than larger ones: although they could still inhibit > 90% of pupal infections within 5 d of unpacking, pupae tested for infection after 1 d still sporulated 70% of the time (Figure 3 – Figure supplement 1; GLM: LR $\chi^2 = 35.23$, $P = 0.001$; Tukey post hoc comparisons: 0 vs. 1 d, $P = 0.2$; 0 vs. 5 d, $P = 0.001$; 1 vs. 5 d, $P = 0.002$). As the effectiveness of destructive disinfection increased with the amount of time the ants had, as well as with the number of ants present, we inferred that there must be a limiting factor affecting the inhibition the pathogen.

To study the underlying mechanisms of destructive disinfection, we performed its different components – unpacking, biting and poison spraying – *in vitro* to test for their relative importance and potential synergistic effects. We simulated unpacking by removing the cocoons of the pupae manually, and the cuticle damage caused by biting using forceps. Previous work establishing the composition of *L. neglectus* poison [55] allowed us to create a synthetic version for use in this experiment (60% formic acid and 2% acetic acid, in water; applied at a dose equivalent to what ants apply during destructive disinfection; Figure 3 – Figure supplement 2), with water as a sham control. We then performed these 'behaviours' in different combinations in a full-factorial experiment. We found that all three behaviours must be performed in the correct order and interact to prevent pathogen replication (overview graph showing odds ratios of sporulation in Figure 3B, full data dataset displayed in Figure 3 – Figure supplement 3; GLM: overall LR $\chi^2 = 79.9$, df = 5, $P = 0.001$; interaction between behaviours LR $\chi^2 = 20.6$, df = 2, $P = 0.001$; all post hoc comparisons in Table 3). As in sanitary care, the poison was the active antimicrobial compound that inhibited fungal growth (Figure 3 – Figure supplement 3, Table 3 [55,144]). However, for the poison to function the pupae had to be removed from their cocoons and their cuticles damaged. Firstly, this is

because the cocoon itself is hydrophobic and thus prevents the aqueous poison from reaching the pupae inside (Figure 3 – Figure supplement 4). Secondly, as the infection is growing internally at the time of unpacking, the cuticle must be broken in order for the poison to enter the hemocoel of the pupae. This is achieved with the perforations created by the ants biting the pupal cuticle. As the active antimicrobial component, we concluded that the poison is probably the limiting factor determining whether destructive disinfection is successful. Because the poison has a slow biosynthesis and each ant can only store a limited amount [55,157], it would explain why destructive disinfection was more likely to be successful the longer the ants had to treat the pupae, and as the number of ants increased (Figure 3A, Figure 3 – Figure supplement 1). By sharing the task of poison synthesis and application, the ants probably increase their chances of preventing the pathogen becoming infectious.

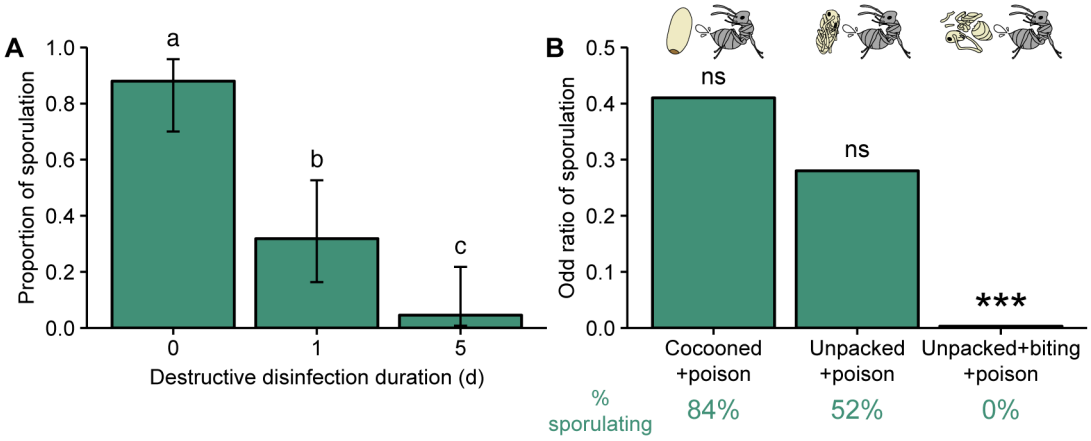

Figure 3. Destructive disinfection by ants prevents pathogen replication.
(A) Destructive disinfection greatly reduced the probability of pupae sporulating compared to pupae that received no destructive disinfection (time point 0), and its effectiveness increased with the length of time ants could perform destructive disinfection (1 vs. 5 d; error bars show ± 95% CI; letters denote groups that differ significantly in Tukey post hoc comparisons [$P < 0.05$]). (B) The individual components of destructive disinfection (unpacking, biting and poison spraying) interacted to inhibit pathogen replication (% of pupae sporulating in each treatment shown under graph in green). The odds of sporulation for cocooned and unpacked pupae treated with poison were not significantly different to those of control pupae (cocooned pupae treated with water). But when unpacking, biting and poison spraying were combined the odds of sporulation were significantly reduced (logistic regression; ns = non-significant deviation from control, *** = $P < 0.001$; complete data set of full factorial experiment displayed in Figure 3 – Figure supplement 3 and all statistics in Table 3).

3.2.4 Disruption of the pathogen lifecycle stops disease transmission

Finally, we investigated the impact of destructive disinfection on disease transmission within a social group. We created mini-nests comprising two chambers and a group of ants (5 ants per group). Into one of the chambers we placed an infectious sporulating pupa – simulating a failure of the ants to detect and destroy the infection – or a pupa that had been destructively disinfected, and was thus non-infectious. The ants groomed, moved around and sprayed both types of corpses with poison. In the case of the sporulating pupae, all conidiospores were removed from the corpse by the ants. As in previous studies, sporulating corpses were highly virulent [202,203] and caused lethal infections that became contagious after host death in 42% of ants (Figure 4A). However, there was no disease transmission from destructively disinfected pupae (Figure 4A; GLM: LR $\chi^2 = 31.32$, df = 1, $P = 0.001$). We therefore

concluded that by preventing the pathogen from completing its lifecycle destructive disinfection stops intra-colony disease transmission (Figure 4B).

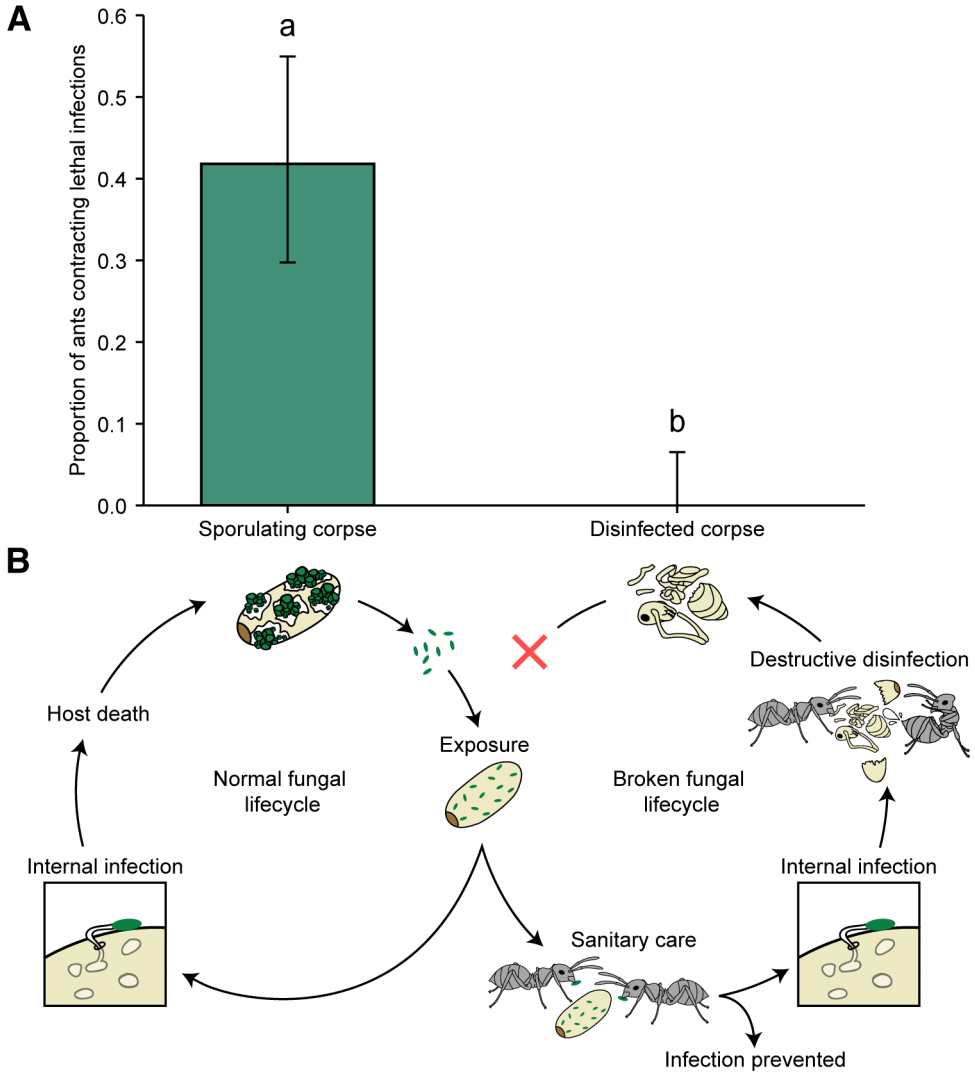

Figure 4. Destructive disinfection stops disease transmission.
(A) Ants that interacted with sporulating pupae contracted lethal infections and died from fungal infection in 42% of the cases, whilst there was no disease transmission from destructively disinfected pupae (error bars show ± 95% CI; letters denote groups that differ significantly in a logistic regression [$P < 0.05$]). (B) Overview of normal fungal lifecycle resulting in infectious, sporulating corpses (left) and a broken lifecycle due to the interference of the ants (right). When sanitary care fails to prevent infection in pathogen-exposed individuals, the ants switch to colony-level disease control, i.e. destructive disinfection to stop pathogen replication, resulting in non-infectious corpses.

3.3 Discussion

Ants are extremely hygienic and frequently perform sanitary behaviours to prevent microbial infection of themselves and colony members [17]. However, if these behaviours fail, the colony faces a problem because infections can become highly contagious and cause disease outbreaks [202,203]. In this study, we have characterised a multicomponent behaviour that

ants use to fight lethal infections of a common fungal pathogen. Our results show that ants detect infected pupae using chemical signatures whilst the pathogen is still in its non-transmissible incubation period (Figure 2). In contrast to the simple removal of infected brood in honeybees [15], the ants then performed destructive disinfection, utilising their antimicrobial poison for internal disinfection of the host body to stop the pathogen from replicating and completing its lifecycle (Figure 1, Figure 3). Ultimately, this prevented the fungus from infecting new hosts and effectively reduced its fitness to zero (Figure 4). These findings extend our current understanding of collective disease defence in ants, showing that they not only avoid [18], groom [20–22] and isolate pathogens [22,26], but can even interfere with the infectious cycle of the pathogen to actively arrest its establishment and replication in the colony (Figure 4b). This will have important implications for the evolution of host-pathogen interactions in social insects, as the pathogen is unable to reproduce. More generally, our results reveal the remarkable adaptations that can evolve in superorganisms to avoid disease outbreaks.

We found that destructive disinfection acts like a second line of defence for the colony, when the first, sanitary care, fails to prevent infection. This has parallels to the immune system of the body where defences are layered to prevent pathogen establishment and replication at multiple levels [51]. The first line of defence in the body is made up of mechanical and chemical defences, such as ciliated cells in the lung that move pathogens trapped in mucus out of the body [51]. In ants, grooming and chemical disinfection during sanitary care play an analogous role [55,144,146]. However, if a pathogen circumvents these defences and a cell is infected, the second line of defence is often a targeted elimination of the cell. This starts with immune cells detecting an infection and then transporting cell death-inducing and antimicrobial compounds into the infected cell by creating pores in its membrane [207–209]. Likewise, our experiments revealed that ants detect sick pupa using chemical compounds on their cuticle. They then unpack the pupa and make perforations in its cuticle, enabling the ants to spray their poison directly into the pupa's body. In both cases, the second line of defence destroys the infected cell/insect, along with the infection, to prevent transmission [210]. Since the loss of somatic cells and individual insect workers can be tolerated with negligible effects on fitness [51], these analogous strategies are a unique way to clear infections and avoid any further damage to the body and colony, respectively.

Previous studies have suggested that ants might use chemical cues to detect sick colony members, but evidence to support this hypothesis has been lacking [105,150,211]. To our knowledge, we have therefore discovered the first known instance of ants using chemical information to identify and specifically target infected individuals. The chemical compounds with increased abundances on infected pupae are distinct from those that induce the removal of corpses in ants [139,142,212], and, like in tapeworm-infected ants [213], are not pathogen-derived because they are also present in lower amounts on healthy pupae. This alteration of the hosts' chemical profile may arise during infection from the breakdown of hydrocarbons by *Metarhizium* penetration [214] or after infection due to an immune response affecting the synthesis of specific hydrocarbons [128,129]. The latter is more likely as the ants only display destructive disinfection once the fungus is growing inside the pupae. Interestingly, two of the four CHCs that were increased on infected pupae also had higher abundances on virus-infected honeybees (Tritriacontadiene [108]) and their brood experiencing a simulated bacterial infection (Tritriacontene [129]). As these compounds belong to the same hydrocarbon substance class – unsaturated hydrocarbons – their common biosynthetic pathway might be upregulated upon infection. This raises the possibility that these hydrocarbons are evolutionarily conserved sickness cues in Hymenopteran social insects. Such cues may have evolved into general sickness signals in social insects as they alert

nestmates to the presence of an infection that will harm the colony if it spreads [215]. Similar to the "find-me/eat-me" signals expressed by infected cells in a body [130,216], they will be selected for as they enhance colony fitness (and hence the indirect fitness of the sick individual) by preventing a systemic infection. Therefore, altruistic displays of sickness can evolve in superorganisms, even if this results in the destruction of the individual that expresses them.

It is well established that social insects use glandular secretions with antimicrobial properties as external surface disinfectants [217]. However, because these compounds can also harm the host, they should be used with caution inside the colony. For example, the acidic poison *L. neglectus* and other Formicine ants produce is extremely caustic and is used to attack conspecifics [55,157,218]. During sanitary care they apply this poison via grooming because it is probably more accurate and less wasteful than spraying [55]. Moreover, as pathogen-exposed insects typically survive when they receive sanitary care [55,144,146,202], conservatively applying the poison may also reduce the damage it causes to individuals that can then continue contributing to the colony. This is supported by our observation that *L. neglectus* will apply large quantities of poison onto pupae only when they become infected. Remarkably, we found that, in addition to being external disinfectants, ants use antimicrobial secretions as internal disinfectants against infections within the bodies of nestmates. Since infected pupae are moribund there is no risk that the ants' poison is harming individuals with a future role in the colony. Taken together, these observations suggest that ants adjust their behaviours in response to the risk presented to the colony. It would be interesting to explore further how social immunity defences are regulated to prevent collateral damage, or 'social immunopathology', within the colony.

Our experiments show that destructive disinfection was highly effective and prevented 95% of infections becoming transmissible. Destructive disinfection will thus keep the average number of secondary infections caused by an initial infection small and the disease will die out within the colony [8]. This may explain why infections of *Metarhizium* and other generalist entomopathogenic fungi like *Beauveria*, though common in the field [46–48,219], do not seem to cause colony-wide epidemics in ants, but are more numerous in solitary species that lack social immunity [85,220,221]. Behaviours like destructive disinfection that are able to reduce pathogen fitness to zero could have selected for host manipulation in fungi that specialise on infecting ants, e.g. *Ophiocordyceps* and *Pandora* [104,222,223]. These fungi force their ant hosts to leave the nest and climb plant stems near foraging trails. There they die and become infectious, releasing new spores that infect ants foraging below. However, ants infected with *Ophiocordyceps* that were experimentally placed back into the nest disappeared [104]. Our study suggests that these ants could have been eliminated through destructive disinfection. Consequently, ant-specialist fungi like *Ophiocordyceps* and *Pandora* may have evolved host manipulation as a means to complete their lifecycle outside of the nest and avoid destructive disinfection [104,223]. In contrast to specialists, generalist pathogens like *Metarhizium* infect a broad range of solitary and social hosts, making it less likely that they evolve strategies to escape social immunity defences [224]. Future work that investigates how social immunity disrupts typical host-pathogen dynamics will shed light on the co-evolution of pathogens and their social hosts [8].

Destructive disinfection has probably evolved in ants because the removal of corpses from the colony alone does not guarantee that disease transmission is prevented [104]. This is because ants place corpses onto midden (trash) sites that are located inside or outside near the nest and regularly visited by midden workers [140,225,226]. Consequently, midden sites

represent a potential source for disease transmission back into the colony. In contrast to ants, honeybees have no middens and corpses are dumped randomly outside of the hive [97]. But because honeybees forage on the wing, it is unlikely that corpses are re-encountered and so removal is sufficient to prevent disease transmission [111]. Termites on the other hand perform a different behaviour, whereby the dead are cannibalised [109,201]. Cannibalism is effective because the termite gut neutralises ingested pathogens [227–229] and has likely evolved because dead nestmates are a source of valuable nitrogen in their cellulose-base diet [100]. The same selective pressure has driven this suite of independently evolved innovations – the need to eliminate or remove infected individuals early in the infectious cycle – with the ants expressing a particularly complex behavioural repertoire. This seems to be a general principle in disease defence as cells are also rapidly detected and destroyed shortly after infection to prevent pathogen spread in multicellular organisms [51]. Understanding how natural selection can result in similar traits at different levels of biological organisation and in organisms with different life histories is a central question in evolutionary biology [50]. Studying the similarities and differences between organismal immunity and social immunity could therefore lead to new insights about how disease defences evolve [51]. For example, the results of our study suggest that equivalent selection pressures can result in convergent defences that protect multicellular organisms and superorganismal insect societies from systemic disease spread. Future work that can link the performance of social immunity defences to colony fitness will therefore provide useful insights into how such traits are selected for over evolutionary time.

3.4 Materials and Methods

Ant host

We studied the unicolonial invasive garden ant, *Lasius neglectus*, collected in Seva, Spain (41.809000, 2.262194) [219]. Stock colonies were kept at a constant temperature of 23°C with 70% humidity and a day/night cycle of 14/10 h. All experiments were conducted in plastered petri dishes (Ø = 33, 55 or 90 mm) with 10% sucrose solution provided *ad libitum* and environmental conditions were controlled throughout (23°C; 70% RH; 14/10 h light/dark cycles). The animal use protocol was performed in accordance with the IST Austria Ethics Committee. At present, the committee does not provide a specific approval numbers for invertebrate animal research. Animals used in this study, *Lasius neglectus*, do not belong to regulated or protected species.

Fungal pathogen

As a model pathogen, we used the entomopathogenic fungus *Metarhizium brunneum* (strain MA275, KVL 03-143). Multiple aliquots were kept in long-term storage at – 80°C. Prior to each experiment the conidiospores were grown on sabaroud dextrose agar at 23°C until sporulation and harvested by suspending them in 0.05% sterile Triton X-100 (Sigma). The germination rate of conidiospore suspensions was determined before the start of each experiment and was > 90% in all cases.

Pupal pathogen exposure

Conidiospores were applied in a suspension of 0.05% autoclaved Triton-X 100 at 10^6 conidia/ml in all experiments unless otherwise stated. Throughout the study, we used cocooned worker pupae of approximately the same age, which was determined by assessing the melanisation of the eyes and cuticle. Single pupae were exposed by gently rolling them in 1 µl of the conidiospore suspension using sterile soft forceps. Pupae were then allowed to air

dry for 5-10 min before being used in experiments. This exposure procedure resulted in pupae receiving ~ 1800 conidiospores, of which 5% (~ 95 conidiospore) passed through the cocoon and came into contact with the pupa inside (Figure 1 – Figure supplement 1).

Statistical Analysis

Statistical analyses were carried out in R version 3.3.2 [230] and all tests were two-tailed. All General(ised) linear and mixed models were compared to null (intercept only) and reduced models (for those with multiple predictors) using Likelihood Ratio (LR) tests to assess the significance of predictors [231]. We controlled for the number of statistical tests performed per experiment to protect against a false discovery rate using the Benjamini-Hochberg procedure ($\alpha = 0.05$). Moreover, all post hoc analyses were corrected for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.05$) [232,233]. We checked the necessary assumptions of all tests i.e. by viewing histograms of data, plotting the distribution of model residuals, checking for non-proportional hazards, testing for unequal variances, testing for the presence of multicollinearity, testing for over-dispersion, and assessing models for instability and influential observations. For mixed effects modelling, we used the packages 'lme4' to fit models [234], 'influence.ME' to test assumptions [235], and, for LMERs, 'lmerTest' to obtain *P* values [236]. All logistic regressions were performed using either generalised linear models (GLMs) or generalised linear mixed models (GLMMs), which had binomial error terms and logit-link function. The Cox proportional hazards regression was carried out using the 'coxphf' package with post hoc comparisons achieved by re-levelling the model and correcting the resulting *P* values [237]. For Kruskal-Wallis (KW) tests and subsequent post hoc comparisons we used the 'agricolae' package, which implements the Conover-Iman test for multiple comparisons using rank sums [238]. For the perMANOVA, we used the package 'vegan' and performed pairwise perMANOVAs for post hoc comparisons [239]. All other post hoc comparisons were performed using the 'multcomp' package [240]. All graphs were made using the 'ggplot2' package [241]. Preliminary studies were performed for all major experiments to determine sample size. No data outliers were detected or removed and all replicate information represents biological replicates. Individual descriptions of statistical analyses are given for all experiments below.

Unpacking behaviour

To study how ants respond to infections, we exposed pupae to a low (10^4/ml), medium (10^6/ml) or high (10^9/ml) dose of conidiospores or autoclaved Triton X as a sham control (sham control, $n = 24$; all other treatments, $n = 25$). The pupae were then placed into individual petri dishes with two ants and inspected hourly for 10 h/d for 10 d. When the ants unpacked a pupa, it was removed and surface-sterilised [242] to ensure that any fungal outgrowth was the result of internal infections and not residual conidiospores on the cuticle. After sterilisation, we transferred the pupae to a petri dish lined with damp filter paper at 23°C and monitored them for 2 weeks for *Metarhizium* sporulation to confirm the presence of an internal infection (low dose, $n = 8$; medium dose, $n = 18$; high, $n = 21$). In addition, any cocooned pupae that were not unpacked after 10 d were removed from the ants, surface sterilised and observed for sporulation, as above (low dose, $n = 11$; medium dose, $n = 4$; high, $n = 4$). We analysed the effect of treatment on unpacking using a Cox proportional hazards model with Firth's penalized likelihood, which offers a solution to the monotone likelihood caused by the complete absence of unpacking in the sham control treatment. We followed up this analysis with post hoc comparisons (model factor re-levelling) to test unpacking rates between treatments (Figure 1B). We compared the number of unpacked and cocooned pupae sporulating using a logistic regression, which included pupa type (cocooned, unpacked), conidiospore dose (low, medium, high) and their interaction as main effects. The interaction

was non-significant (GLM: LR χ^2 = 5.0, df = 2, P = 0.084); hence, it was removed to gain better estimates of the remaining predictors.

Images and scanning electron micrographs (SEMs) of destructive disinfection
Photographs of destructive disinfection were captured (Nikon D3200) and aesthetically edited (Adobe Photoshop) to demonstrate the different behaviours (Figure 1A). They were not used in any form of data acquisition. We also made representative SEMs of a pupa directly after unpacking and one after destructive disinfection (24 h after unpacking; Figure 1F-G). As the pupae were frozen at – 80ºC until the SEMs were made, we also examined non-frozen pupae taken directly from the stock colony and confirmed that freezing itself does not cause damage to the pupa (not shown).

Conidiospore load on unpacked pupae
We determined the number of conidiospores on unpacked pupae (n = 7) and their removed cocoons (n = 7) by placing them into separate vials containing 100 µl autoclaved 0.05 % Triton-X 100. The vials were then shaken for 10 m at 600 RPM (Vortex Genie 2) and the resulting supernatant was plated onto selective medium agar. We counted the number of *Metarhizium* colony forming units (CFUs) that subsequently grew on the plates after 7 d. As a control, we performed the same experiment on pupae directly after pathogen-exposure. We experimentally unpacked the pupae using forceps so that we could examine the number of CFUs present on the pupae (n = 16) and cocoon separately (n = 16). We analysed the number of CFUs on pupae and cocoons using Mann-Whitney U-tests (Figure 1 – Figure supplement 1).

Comparison of sanitary care and destructive disinfection behaviours
To observe how the behavioural repertoire of the ants changes between sanitary care and destructive disinfection, we filmed three individually colour-marked ants tending a single pathogen-exposed pupa with a USB microscope camera (Di-Li ® 970-O). To characterise the sanitary care behaviours of the ants, we analysed the first 24 h of the videos following the introduction of the pupa. To study destructive disinfection behaviours, we analysed the 24 h period that immediately followed unpacking. Videos were analysed using the behavioural-logging software JWatcher™ [243]. For each ant (n = 15), we recorded the duration of its grooming bouts, the frequency of poison application and the frequency of biting. Grooming duration was analysed using a LMER, having first log-transformed the data to fulfil the assumption of normality (Figure 1C). The frequency of poison spraying and biting (Figure 1D-E) were analysed using separate GLMMs with Poisson error terms for count data and logit-link function. We included an observation-level random intercept effect to account for over-dispersion in the poison spraying and biting data [244]. In all three models, we included petri dish identity as a random intercept effect because ants from the same dish are non-independent. Additionally, a random intercept effect was included for each ant as we observed the same individuals twice (before and after unpacking).

Comparison of pupal mortality after unpacking and destructive disinfection
We established a protocol to determine whether pupae were dead or alive because it is not generally obvious when death has occurred. To ensure that we examined pupae as soon as possible after unpacking, we checked pathogen-exposed pupae housed with ants every 45 min for 15 h/d. When unpacking occurred, we either removed the pupa immediately (n = 33) or left it with the ants for a further 24 h so that they could perform destructive disinfection (n = 44). To check the numbers of dead and alive pupae at the time point of unpacking and after destructive disinfection, we secured the pupae to glass slides using double-sided tape. The

pupae were then gently prodded with a glass capillary whilst being examined under a bifocal microscope (10 x magnification; Leica DM 1000). If pupae were alive, this resulted in contractions of their dorsal aorta [245], which is visible through the cuticle of the abdomen. If they were dead, no contractions occurred. Each examination lasted a maximum of 5 min. To confirm that this approach was sensitive, we examined experimentally unpacked pupae taken straight from a stock colony (n = 10). In all cases, these pupae were alive. They were then frozen at – 80 ºC for 1 d and examined again after defrosting, when they were all found to be dead. We compared the number of dead pupae at the time point of unpacking to the number that were dead after destructive disinfection using a logistic regression (Figure 1 – Figure supplement 2). We included the day of unpacking as a covariate to test if pupae unpacked sooner or later were more or less likely to have already died.

Estimation of poison load on pupae after destructive disinfection

As *L. neglectus* poison has a very low acidity [55], we could measure the pH of pupae to determine if ants apply higher amounts poison to pupae during destructive disinfection. We kept a pair of pathogen-exposed or sham control pupae with two ants. When one of the pathogen-exposed pupae in a pair was unpacked, we let the ants perform destructive disinfection for 24 h (n = 25). In the control, we experimentally unpacked one pupa in a pair and placed it back with the ants for 24 h (n = 17). After 24 h, we removed the unpacked pupae in both treatments along with their discarded cocoons. At the same time, the second, still cocooned pupae in each pair was removed and experimentally unpacked so that pH measurements were consistent across pupal groups (pathogen exposed, n = 9; control, n = 16). All pupae and their cocoons were placed into individual vials containing 20 µl of autoclaved distilled water and a sterile glass pestle was used to crush each pupa and cocoon for 60 s. The pH of the resulting pupa/cocoon slurry was measured using a pH electrode meter (INLAB ULTRA-MICRO, SevenGo PRO pH SG8 pH-meter; Mettler-Toledo). This gave us an indication of how much poison the ants had applied to each type of pupa (Figure 1 – Figure supplement 3). We used a LMER with Tukey post hoc comparisons to compare the pH measurements of the pupae. Pupa treatment (pathogen-exposed or control), type (cocooned or unpacked) and their interaction were included as main effects. Petri dish was included as a random intercept effect as pairs of pupae from the same dish are non-independent. As we used a portion of this dataset in Figure 3 – Figure supplement 2, we corrected the overall model P value for multiple testing.

Chemical bioassay

We determined whether ants detect infected pupae through potential changes in the pupae's cuticular chemical profile. We established internal infections in pupae by exposing them to the pathogen and leaving them for 3 d in isolation. In pilot studies, approx. 50% of these pupae were then unpacked within 4 h of being introduced to ants. After 3 d, pupae were washed for 2.5 min in 300 µl of either pentane solvent to reduce the abundance of all CHCs present on the pupae (n = 28), or in autoclaved water as a handling control (n = 28). After washing, pupae were allowed to air dry on sterile filter paper. Additionally, non-washed pupae were used as a positive control (n = 30). Pupae were placed individually with a pair of ants in petri dishes and observed for unpacking for 4 h. We used GC–MS (see below for methodology) to confirm that washing was effective at removing cuticular compounds, by comparing the total amount of chemicals present on pupae washed in pentane to non- and water-washed pupae (n = 8 per treatment; Figure 2 – Figure supplement 1). The number of pupae unpacked between the different treatments was analysed using a logistic regression (Figure 2A). As several researchers helped to wash the pupae, we included a random intercept for each person to control for any potential handling effects. Additionally, the

experiment was run in two blocks on separate days, so we included a random intercept for each block to generalise beyond any potential differences between runs. The total peak area from the GC–MS analysis was compared between treatments using a KW test with post hoc comparisons.

Chemical analysis of pupal hydrocarbon patterns

To confirm that infected pupae had chemical profiles that are different from pathogen-exposed cocooned and control pupae, we exposed pupae to the pathogen or a sham control. Pupae were then isolated for 3 d to establish infections in the pathogen-exposed treatment (as above). Following isolation, pupae were individually placed with ants and observed for unpacking for 4 h. Unpacked pupae were immediately frozen at – 80 °C with the removed cocoons (n = 13) and we also froze cocooned pathogen-exposed pupa that had not yet been unpacked (n =10). Furthermore, we froze a pair of control pupae, of which one was cocooned (n = 12), whilst the other was first experimentally unpacked (to test if the cocoon affects cuticular compound extraction; n =12). Cuticular chemicals were extracted from individual pupae and their cocoons in glass vials (Supelco; 1.8 ml) containing 100 µl n-pentane solvent for 5 min under gentle agitation. The vials were then centrifuged at 3000 rpm for 1 min to spin down any fungal conidiospores that might be remaining, and 80 µl of the supernatant was transferred to fresh vials with 200 µl glass inserts and sealed with Teflon faced silicon septa (both Supelco). The pentane solvent contained four internal standards relevant for our range of hydrocarbons (C_{27} – C_{37}); n-Tetracosane, n-Triacontane, n-Dotriacontane and n-Hexatriacontane (Sigma Aldrich) at 0.5 µg/ml concentration, all fully deuterated to enable spectral traceability and separation of internal standards from ant-derived substances. We ran extracts from the different groups in a randomised manner, intermingled with blank runs containing only pentane, and negative controls containing the pentane plus internal standards (to exclude contaminants emerging e.g. from column bleeding), on the day of extraction, using GC–MS (Agilent Technologies; GC7890 coupled to MS5975C).

A liner with one restriction ring filled with borosilicate wool (Joint Analytical Systems) was installed in the programmed temperature vaporisation (PTV) injection port of the GC, which was pre-cooled to -20 °C and set to solvent vent mode. 50 µl of the sample extractions were injected automatically into the PTV port at 40 µl/s using an autosampler (CTC Analytics, PAL COMBI-xt; Axel Semrau, CHRONOS 4.2 software) equipped with a 100 µl syringe (Hamilton). Immediately after injection, the PTV port was ramped to 300 °C at 450 °C/min, and the sample transferred to the column (DB-5ms; 30 m × 0.25 mm, 0.25 µm film thickness) at a flow of 1 ml/min. The oven temperature program was held at 35 °C for 4.5 min, then ramped to 325 °C at 20 °C/min, and held at this temperature for 11 min. Helium was used as the carrier gas at a constant flow rate of 3 ml/min. For all samples, the MS transfer line was set to 325 °C, and the MS operated in electron ionisation mode (70 eV; ion source 230 °C; quadrupole 150 °C, mass scan range 35-600 amu, with a detection threshold of 150). Data acquisition was carried out using MassHunter Workstation, Data Acquisition software B.07.01 (Agilent Technologies).

Analytes were detected by applying deconvolution algorithms to the total ion chromatograms of the samples (MassHunter Workstation, Qualitative Analysis B.07.00). Compound identification (Table 1) was performed via manual interpretation using retention indices and spectral information, and the comparison of mass spectra to the Wiley 9th edition/NIST 11 combined mass spectral database (National Institute of Standards and Technologies). As the molecular ion was not detectable for all analytes based on electronic ionisation, we in addition performed chemical ionisation on pools of 20 pupae in 100 µl n-pentane solvent

with 0.5 µg/ml internal standards. The higher extract concentration was needed to counteract the loss in ionisation efficiency in chemical ionisation mode. A specialised chemical ionisation source with methane as the reagent gas was used with the MS, while the chromatographic method was the same as in electronic ionisation mode. Use of external standards (C_7-C_{40} saturated alkane mixture [Sigma Aldrich]) enabled traceability of all peaks, and thus comparison to runs of single pupae extracts made in electronic ionisation mode. Modified Kovats retention indices for the peaks in question were calculated based on those standards. To further aid identification, we separated the substances based on polarity using solid phase extraction fractionation. For this purpose, pools of 20 pupae were extracted in 500 µl n-pentane containing 0.2 µg/ml internal standard, and separated on unmodified silica cartridges (Chromabond ® SiOH, 1ml, 100 mg) based on polarity. Prior to use, the cartridges were conditioned with 1 ml dichloromethane followed by 1 ml n-pentane. The entire extraction volume was loaded onto the silica and the eluent (fraction 1, highly apolar phase) collected. A wash with 1 ml pure n-pentane was added to fraction 1. Fraction 2 contained all substances washed off the silica with 1 ml 25 % dichloromethane in n-pentane, and finally a pure wash with 1 ml dichloromethane eluted all remaining substances (fraction 3). The polarity thus increased from fraction 1 through 3, but no polar substances were found. All fractions were dried under a gentle nitrogen stream and re-suspended in 70 µl n-pentane followed by vigorous vortexing for 45 s. GC–MS analysis of all fractions was performed in electronic ionisation mode under the same chromatographic conditions as before.

To quantify the relative abundances of all compounds found on each pupa, analyte-characteristic quantifier and qualifier ions were used to establish a method enabling automatized quantification of their integrated peak area relative to the peak area of the closest internal standard. For each analyte, the relative peak area was normalised, i.e. divided by the total sum of all relative peak areas of one pupa, to standardise all pupa samples. Only analytes, which normalised peak area contributed more than 0.05% of the total peak area, were included in the statistical analysis. We compared the chemical profiles of the pupae using a perMANOVA analysis of the Mahalanobis dissimilarities between pupae, with post hoc perMANOVA comparisons. Since there was no difference between cocooned and unpacked control pupae we combined them into a single control group for the final analysis (perMANOVA: $F = 1.09$, df = 23, $P = 0.1$). We also performed a discriminant analysis of principle components (Figure 2B) to characterise the differences between the pupal treatments [246,247]. To identify the compounds that differ between treatments, we performed a conditional random forest classification (*n* trees = 500, *n* variables per split = 4) [246,248,249]. Random forest identified 9 compounds that were important in classifying the treatment group, of which 8 were significant when analysed using separate KW tests (results for significant compounds in Table 2). We followed up the KW tests with individual post hoc comparisons for each significant compound (Figure 2C-F, post hoc comparisons in Table 2).

Effect of destructive disinfection on pathogen replication

To test if destructive disinfection prevents *Metarhizium* from successfully replicating, we kept single pathogen-exposed pupae in petri dishes containing groups of 3 or 8 ants. This allowed us to assess how group size affects the likelihood of fungal inhibition. For the following 10 d, we observed the pupae for unpacking. When a pupa was unpacked, we left it with the ants for a further 1 or 5 d so that they could perform destructive disinfection. This allowed us to assess how the duration of destructive disinfection affects the likelihood of fungal inhibition. The destructively disinfected pupae were then removed and placed into petri dishes on damp filter paper at 23 ºC (8 ants 1 d and 5 d, $n = 22$ pupae each; 3 ants 1 and 5 d, $n = 18$ pupae each). We did not surface sterilise the pupae as this might have interfered

with the destructive disinfection the ants had performed. Removed pupae were observed daily for *Metarhizium* sporulation for 30 d. To determine how many pupae sporulate in the absence of destructive disinfection, we kept pathogen-exposed pupae without ants as a control and recorded the number that sporulated for 30 d (n = 25). We compared the number of pupae that sporulated after 1 d, 5 d and in the absence of ants using logistic regressions and Tukey post hoc comparisons, separately for the two ant group sizes (Figure 3A, Figure 3 – Figure supplement 1).

***In vitro* investigation of destructive disinfection**

We examined the individual effects of unpacking, biting and poison application on destructive disinfection by performing these behaviours *in vitro*. Pathogen-exposed pupae were initially kept with ants so that they could perform sanitary care. After 3 d, we removed the pupae and split them up into three groups: (i) pupae that we left cocooned, (ii) experimentally unpacked and (iii) experimentally unpacked and bitten. We simulated the damage the ants achieve through biting by damaging the pupal cuticle and removing their limbs with forceps. The pupae were then treated with either synthetic ant poison (60% formic acid and 2% acetic acid, in water; applied at a dose equivalent to what ants apply during destructive disinfection; Figure 3 – Figure supplement 2) or autoclaved distilled water as a control, using pressurised spray bottles (Lacor) to evenly coat the pupae in liquid. The pupae were allowed to air dry for 5 min before being rolled over and sprayed again and allowed to dry a further 5 min. All pupae were then placed into separate petri dishes and monitored daily for *Metarhizium* sporulation (cocooned + poison, n = 24; unpacked + poison + biting, n = 24; all other treatments, n = 25). The number of pupae sporulating was analysed using a logistic regression with Firth's penalised likelihood, which offers a solution to the monotone likelihood caused by the complete absence of sporulation in one of the groups (R package 'brglm' [250]). Pupal manipulation (cocooned/unpacked only/unpacked and bitten), chemical treatment (water or poison) and their interaction were included as main effects (Figure 3B, Figure 3 – Figure supplement 3). We followed up this analysis with Tukey post hoc comparisons (Table 3).

Comparing synthetic and ant poison spraying

We confirmed that synthetic poison spraying resulted in pupae receiving an amount of poison within the natural range that is applied by ants during destructive disinfection. Pupae taken from a stock colony were experimentally unpacked and sprayed with synthetic poison. We then measured their pH (all as above; n = 21). To test if synthetic poison spraying was similar to natural ant spraying, we compared their pH to pupae destructively disinfected by ants (data from Figure 1 – figure supplement 3) using a Mann-Whitney U test (Figure 3 – Figure supplement 2). We corrected the P value to correct for using this dataset twice (here and in Figure 1 – Figure supplement 3).

The effect of the pupal cocoon on ant poison application

To test if the pupal cocoon limits the amount of the ants' poison that reaches the pupae inside, we took pupae from a stock colony and sprayed half with synthetic ant poison (as above; n = 10) and left the other half untreated (n = 10). We then unpacked these pupae and measured their pH (as above). As an additional control, we first experimentally unpacked pupae before spraying them with synthetic poison (n = 10). We analysed pH pupae using a KW test with post hoc comparisons (Figure 3 – Figure supplement 4).

Disease transmission from infectious and destructively disinfected pupae

We tested the impact of destructive disinfection on disease transmission within groups of ants by keeping them with sporulating pupae or pupae that had been destructively disinfected. Infections were established in pupae (as above) and half were allowed to sporulate (n = 11), whilst the other half were experimentally destructively disinfected (as above; n = 11). Pupae were then kept individually with groups of 5 ants in mini-nests (cylindrical containers [Ø = 90 mm] with a second, smaller chamber covered in red foil [Ø = 33 mm]). Ant mortality was monitored daily for 30 d. Dead ants were removed, surface sterilised and observed for *Metarhizium* sporulation. The number of ants dying from *Metarhizium* infections in each treatment was compared using a logistic regression (Figure 4A). Mini-nest identity was included as a random intercept effect as ants from the same group are non-independent.

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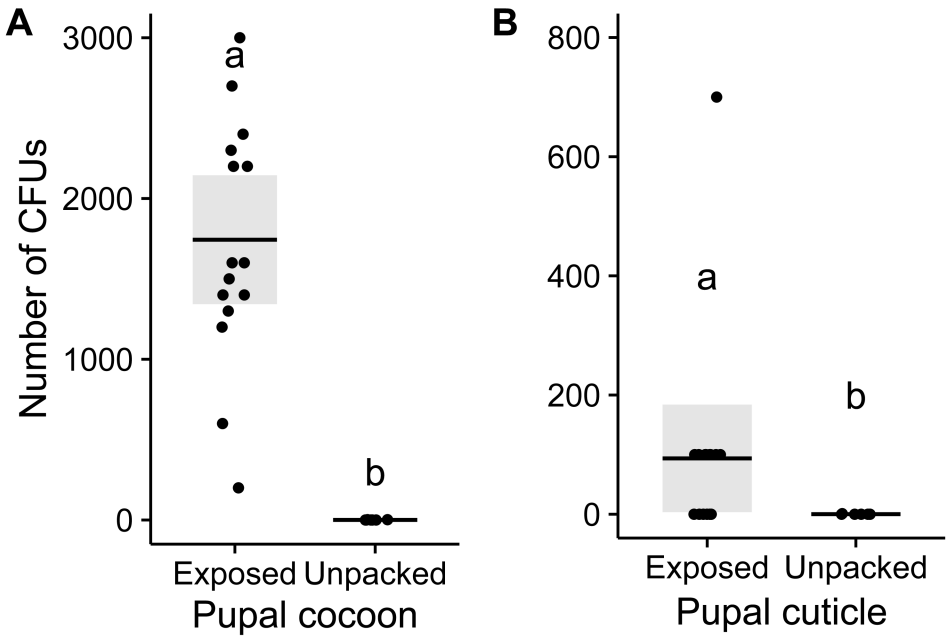

Figure 1 – Figure supplement 1. Conidiospore load on pupae.
We washed pupae to recover conidiospores from their (A) cocoons and (B) cuticles. The washes were then plated and the resultant colony forming units (CFUs) were counted. Whilst we could recover many conidiospores from cocoons and pupae immediately after pathogen exposure, we recovered almost no viable conidiospores from unpacked pupae and their removed cocoons (cocoons: Mann-Whitney *U* test, $U = 112$, $P = 0.001$, pupae: $U = 84$, $P = 0.037$). This indicates that, at the time of unpacking, most conidiospores have been removed and/or chemically disinfected by the ants during sanitary care, or have successfully germinated and caused internal infections within the pupae. All data points displayed; line ± shaded boxes show mean ± 95% CI. Letters groups that are significantly different in Mann-Whitney *U* tests ($P < 0.05$).

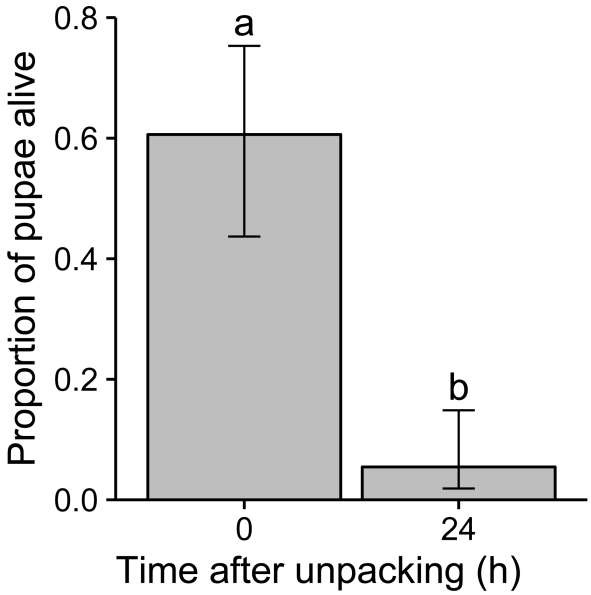

Figure 1 – Figure supplement 2. Unpacked pupae are killed by destructive disinfection.
Unpacked pupae were observed for signs of mortality (observations for contractions of dorsal aorta) immediately after unpacking (0 h) or following destructive disinfection by ants (24 h after unpacking). At the time of unpacking, the majority of pupae were still alive, whilst pupae left with workers for one day were mostly dead. In addition, the later pupae were unpacked the more likely they were to have already died (GLM: overall model LR $\chi^2 = 49.76$, df = 2, $P = 0.001$; 0 vs. 24 h: LR $\chi^2 = 36.28$, df =1, $P = 0.001$; effect of time: LR $\chi^2 = 16.19$, df =1, $P = 0.001$). Error bars show ± 95 % CI. Letters show groups that are significantly different in logistic regression ($P < 0.05$).

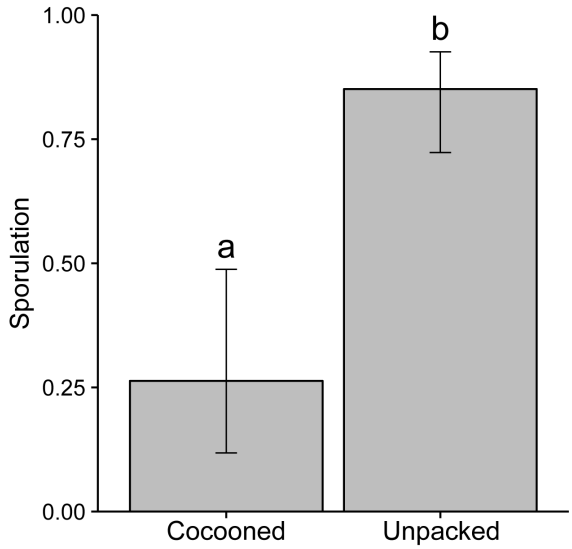

Figure 1 – Figure supplement 3. Unpacked pupae are infected.
Unpacked pupae and pathogen-exposed pupae that were not unpacked (cocooned) were removed from ants and incubated for fungal growth to determine how many were infected. A greater amount of unpacked pupae were infected than those that were not unpacked. Error bars show ± 95 % CI. Letters show groups that are significantly different in logistic regression ($P < 0.05$).

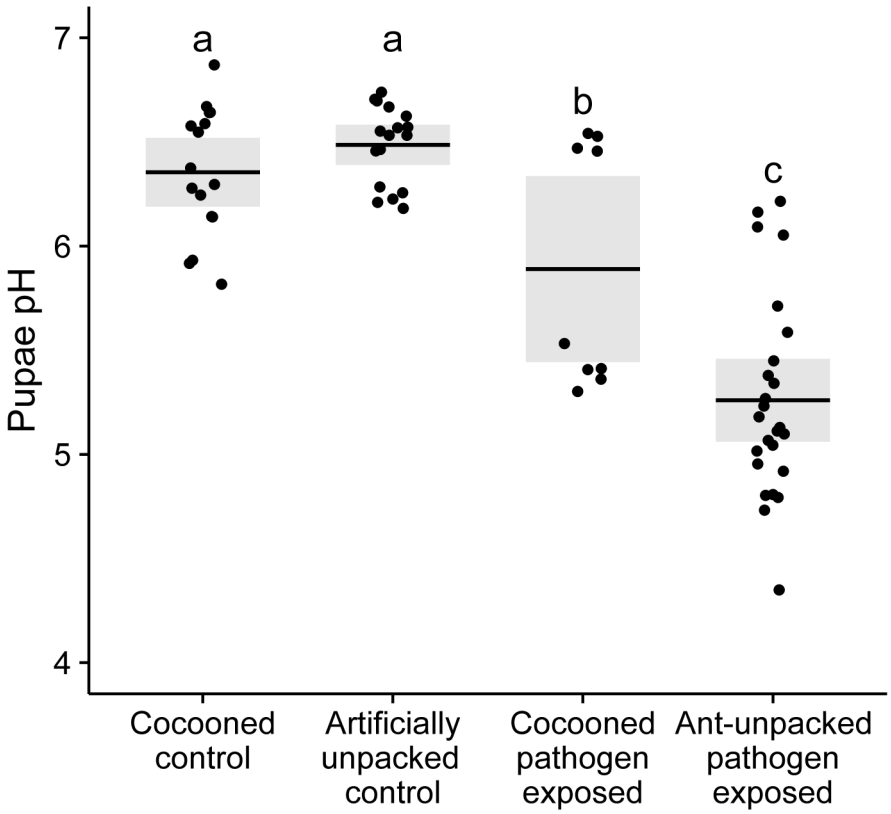

Figure 1 – Figure supplement 4. Destructive disinfection reduces pupa pH.
Pupae that were unpacked by the ants received more poison within the 24 h after unpacking – when ants perform destructive disinfection – compared to non-unpacked pathogen-exposed pupae and both controls (GLM: overall model LR χ^2 = 59.01, df = 3, P = 0.001; interaction between unpacking and treatment: LR χ^2 = 15.42, df = 1, P = 0.001; Tukey post hoc comparisons: cocooned control vs. unpacked control, P = 0.25; cocooned control vs. cocooned pathogen, P = 0.002; all others, P = 0.001). All data points displayed; lines ± shaded boxes show mean ± 95% CI. Letters denote groups that differ significantly in Tukey post hoc comparisons ($P < 0.05$).

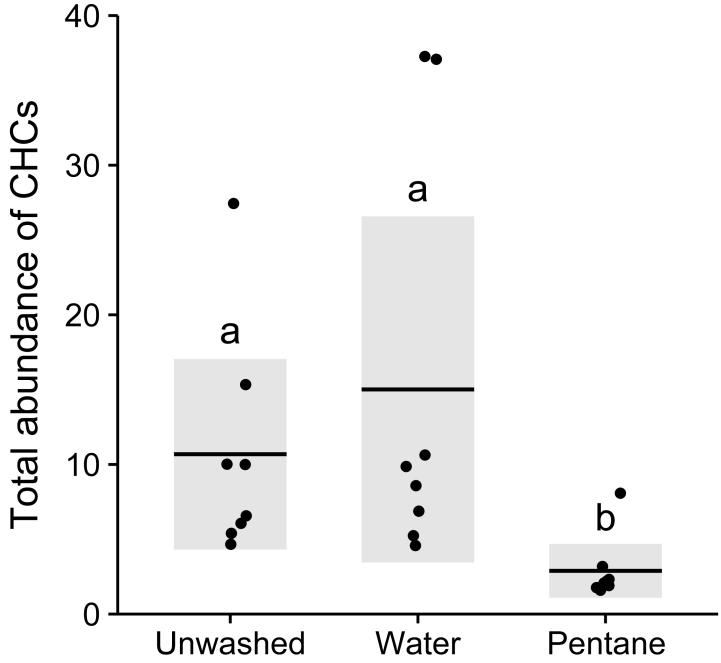

Figure 2 – Figure supplement 1. Total abundance of cuticular hydrocarbons (CHCs) on pupae.
Washing pupae in pentane solvent reduced the total amount of extractable CHCs on pupae, compared to unwashed and water-washed control pupae, confirming that this method is effective at reducing CHC abundance (KW test: $H = 12.26$, df $= 2$, $P = 0.002$; post hoc comparisons: unwashed vs. water, $P = 0.73$; all others, $P = 0.001$). All data points displayed; lines ± shaded boxes show mean ± 95% CI. Letters show groups that differ significantly in post hoc comparisons ($P < 0.05$).

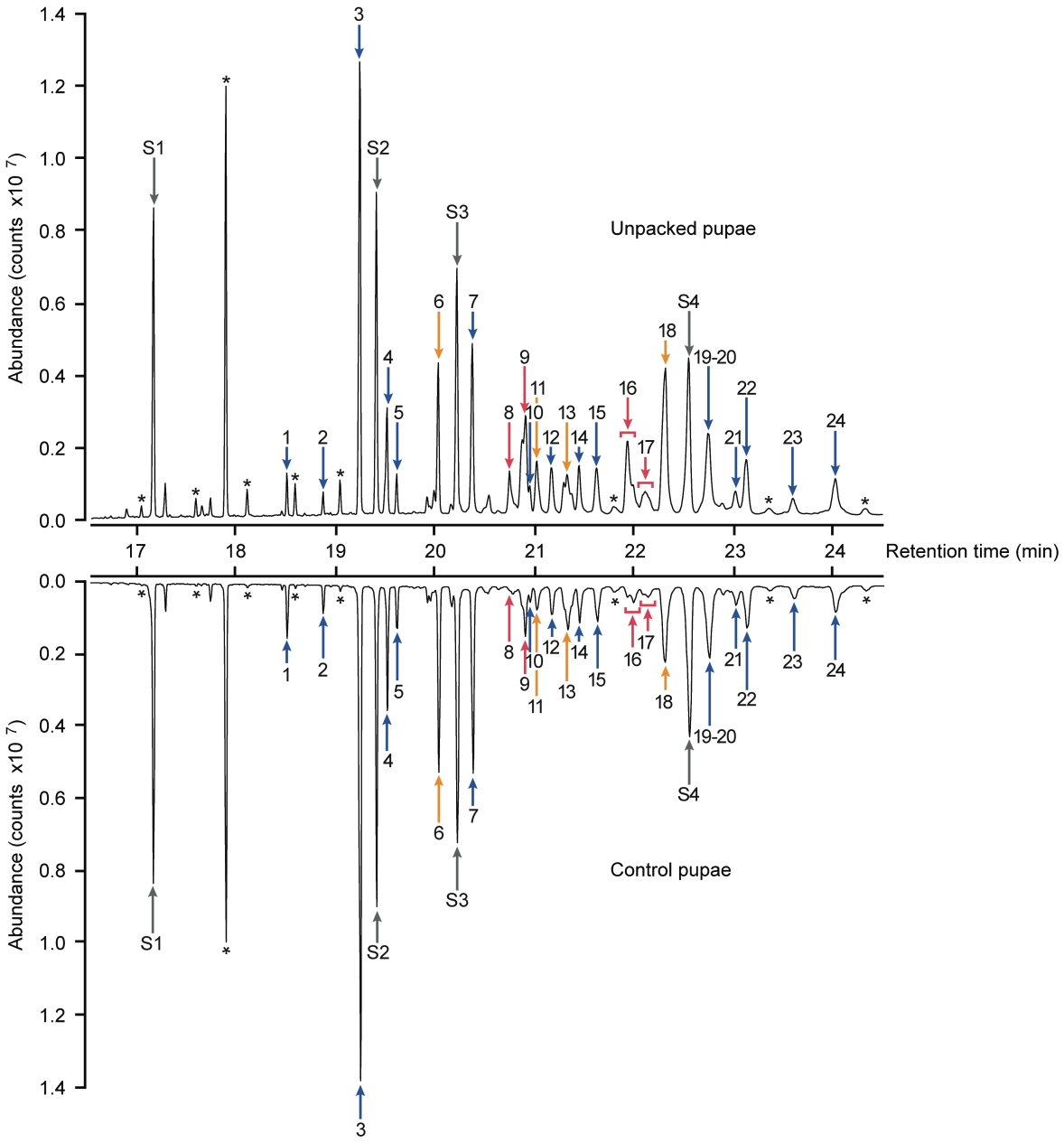

Figure 2 – Figure supplement 2. The cuticular hydrocarbon (CHC) profiles of unpacked and control pupae.

Example total ion chromatograms of an unpacked pathogen-exposed pupa (top) and a sham-treated control pupa (bottom), showing the 24 CHC peaks extracted from the pupae/cocoons (for identifications see Table 1) and the added internal standards S1-4 (grey arrows; S1 = n-Tetracosane-d50; S2 = n-Triacontane-d62; S3 = n-Dotriacontane-d66; S4 = n-Hexatriacontane-d74). Contaminants resulting from column bleeding (e.g. phthalic ester) are marked by asterisks. Red arrows indicate those compounds that showed significantly higher relative peak intensity on pathogen-exposed unpacked pupae, compared to both pathogen-exposed, yet left cocooned, or sham-treated pupae (Table 2). Orange arrows show the peaks that differ between pathogen-exposed unpacked and sham-treated pupae, but not between pathogen-exposed unpacked and cocooned pupae. Blue arrows mark all remaining peaks.

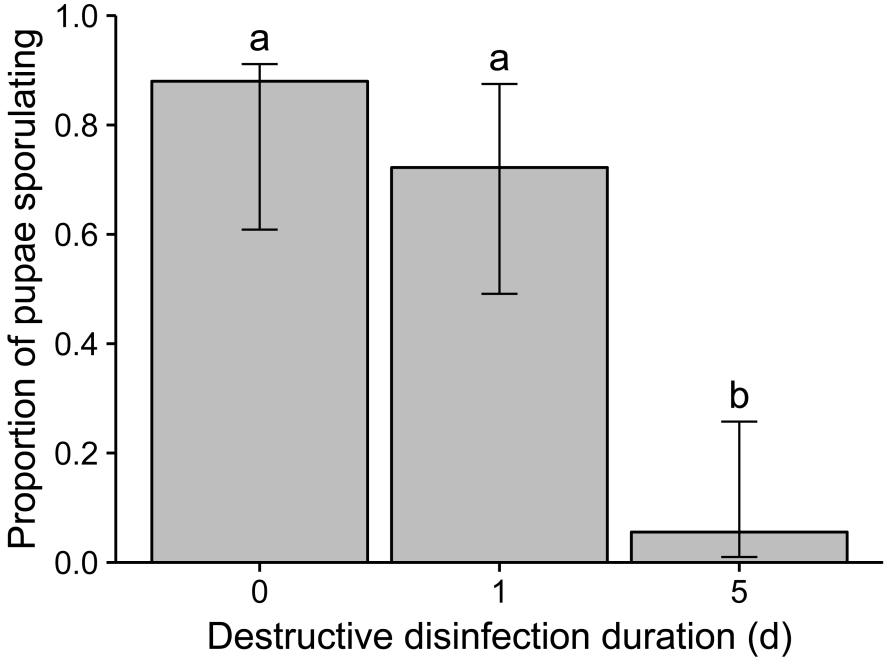

Figure 3 – Figure supplement 1. Destructive disinfection of infected pupae in small groups of ants is less efficient.

Destructive disinfection by groups of 3 ants greatly reduced the probability of pupae sporulating compared to pupae that received no destructive disinfection (time point 0) 5 d after unpacking. However, the proportion of pupae sporulating was equal when ants only had 1 day to perform destructive disinfection, as compared to those that received no destructive disinfection (time point 0). Error bars show ± 95% CI; letters denote groups that differ significantly in Tukey post hoc comparisons ($P < 0.05$).

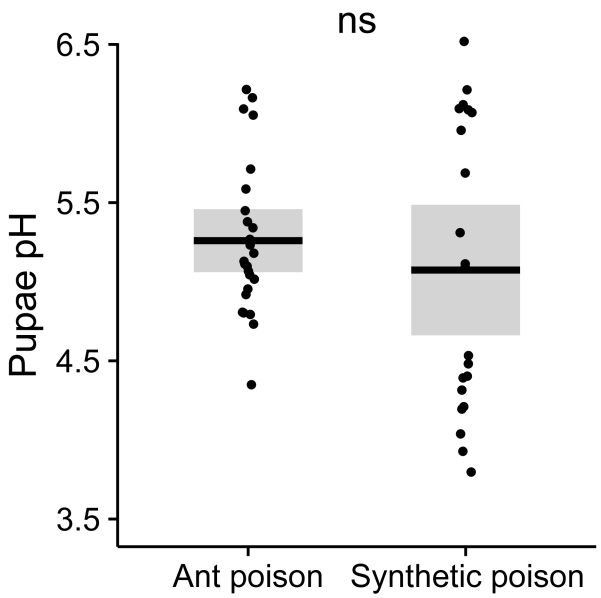

Figure 3 – Figure supplement 2. Comparison of ant and synthetic poison spraying.
Experimental application of synthetic poison (60% formic acid and 2% acetic acid, in water [55]) resulted in pupae receiving quantities of poison similar to pupae kept with ants for one day after unpacking (determined by measuring pupal pH after spraying them with poison and comparing to data from Figure 1 – figure supplement 3; Mann-Whitney *U* test: $U = 303$, $P = 0.38$), meaning we were applying poison to pupae in realistic amounts as compared to the ants and inducing an equivalent decrease in pH change. All data points displayed; lines ± shaded boxes show mean ± 95% CI. Treatments were non-significant (ns) in a Mann-Whitney *U* test ($P > 0.05$).

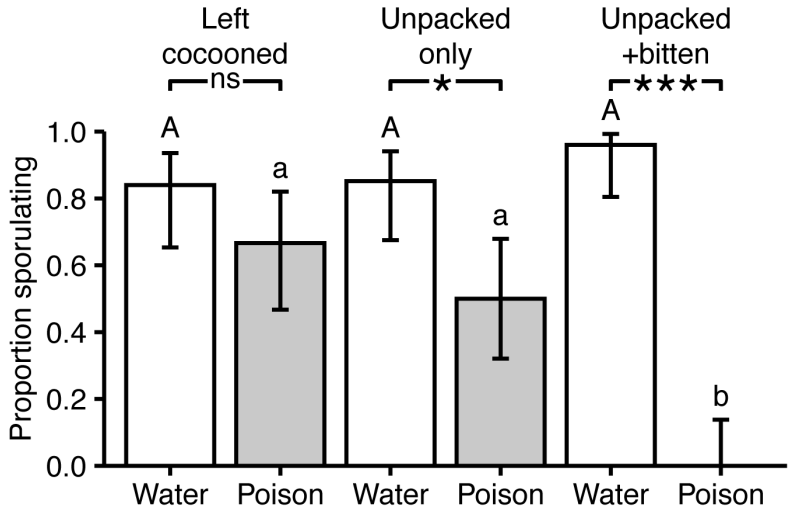

Figure 3 – Figure supplement 3. Destructive disinfection by ants prevents pathogen replication.
Unpacking, biting and poison application interact to inhibit fungal sporulation. Only when all three 'behaviours' are performed is fungal sporulation completely prevented (grey = poison, white = water). Error bars show ± 95% CI. Uppercase letters denote bars that differ ($P < 0.05$) within the water treatment, lowercase letters show differences within the poison treatment, and asterisks indicate differences within pupal groups: ns = non-significant, * = $P < 0.05$, *** = $P < 0.001$ (full post hoc comparisons given in Table 3).

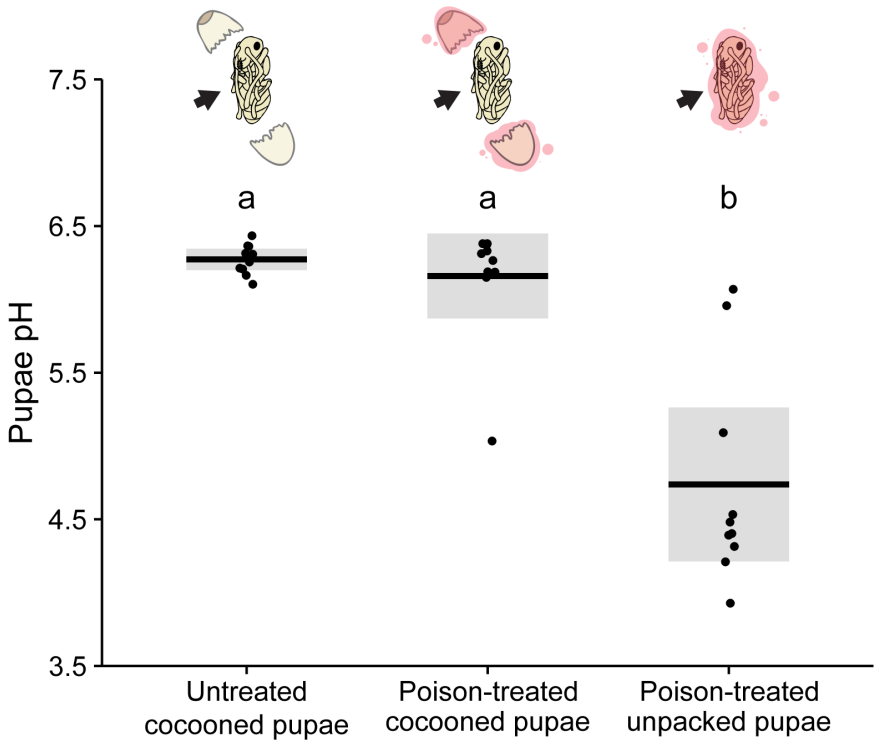

Figure 3 – Figure supplement 4. The pupal cocoon blocks the application of poison.
Cocooned pupae treated with poison had a pH equivalent to untreated cocooned pupae, whereas poison-treated unpacked pupae had a significantly decreased pH, revealing that the cocoon blocks the application of poison (KW test: $H = 18.22$, df = 2, $P = 0.001$; post hoc comparisons: untreated cocooned vs. poison-treated unpacked, $P = 0.91$; all others, $P = 0.001$). All data points displayed; lines ± shaded boxes show mean ± 95% CI. Letters denote groups that differ significantly in post hoc comparisons ($P < 0.05$).

Tables

Table 1. Compound identification of cuticular hydrocarbons (CHCs) from *Lasius neglectus* pupae.
Table shows all 24 CHCs, with peak numbers listed in the order of their retention time, as in Figure 3 – Figure supplement 2. For comparability across gas chromatography–mass spectrometry systems, modified Kovats indices are included. Peaks that were significantly higher on unpacked pupae are highlighted in bold. In peaks 17 and 18, two compounds co-eluted.

Peak #	Compound identification	Retention time (min)	Modified Kovats index
1	n-Heptacosane	18.521	2699
2	n-Octacosane	18.883	2799
3	n-Nonacosane	19.253	2902
4	3-Methylnonacosane	19.529	2974
5	n-Triacontane	19.624	2999
6	n-Hentriacontane	20.040	3100
7	3-Methylhentriacontane	20.387	3175
8	**Tritriacontadiene**	**20.764**	**3251**
9	**Tritriacontene**	**20.910**	**3279**
10	Tritriacontene	20.958	3288
11	n-Tritriacontane	21.019	3300
12	13-Methyltritriacontane	21.174	3326
13	3-Methyltritriacontene	21.335	3353
14	3-Methyltritriacontane	21.456	3373
15	n-Tetratriacontane	21.626	3402
16	**Pentatriacontadiene**	**21.937**	**3447**
17	**Pentatriacontadiene + Pentatriacontene**	**22.134**	**3475**
18	n-Pentatriacontane + 13-Methylpentatriacontene	22.306	3500
19	13,23-Dimethylpentatriacontane	22.740	3554
20	11,25-Dimethylpentatriacontane	22.752	3556
21	7,11,23-Trimethylpentatriacontane	23.019	3589
22	n-Hexatriacontane	23.125	3602
23	Unknown	23.603	3652
24	n-Heptatriacontane	24.023	3697

Table 2. Compounds contributing most to the differences between pupal cuticular hydrocarbon (CHC) profiles.
Table gives the overall effect of treatment per CHC, corrected for multiple testing, and the post hoc comparisons, corrected at the level of each compound for multiple comparisons. CHCs significantly increased specifically on unpacked pupae shown in bold. All multiple comparison corrections performed using the Benjamini-Hochberg procedure ($\alpha = 0.05$).

Peak #	Compound	KW *H* (df = 2)	Corrected KW *P*-value	Post-hoc comparison	Adjusted *P*-value
6	n-Hentriacontane	7.29	0.029	Cocooned – Unpacked	0.238
				Cocooned – Control	0.309
				Unpacked – Control	0.019
8	**Tritriacontadiene**	**13.11**	**0.006**	**Cocooned – Unpacked**	**0.005**
				Cocooned – Control	**0.830**
				Unpacked – Control	**0.001**
9	**Tritriacontene**	**10.39**	**0.01**	**Cocooned – Unpacked**	**0.021**
				Cocooned – Control	**0.745**
				Unpacked – Control	**0.003**
11	Tritriacontane	11.55	0.007	Cocooned – Unpacked	0.064
				Cocooned – Control	0.245
				Unpacked – Control	0.001
14	3-Methyltritriacontene	7.63	0.028	Cocooned – Unpacked	0.428
				Cocooned – Control	0.143
				Unpacked – Control	0.021
16	**Pentatriacontadiene**	**18.83**	**0.001**	**Cocooned – Unpacked**	**0.004**
				Cocooned – Control	**0.152**
				Unpacked – Control	**< 0.001**
17	**Pentatriacontadiene + Pentatriacontene**	**12.09**	**0.007**	**Cocooned – Unpacked**	**0.039**
				Cocooned – Control	**0.301**
				Unpacked – Control	**0.001**
18	n-Pentatriacontane + 13-Methylpentatriacontene	10.12	0.01	Cocooned – Unpacked	0.083
				Cocooned – Control	0.312
				Unpacked – Control	0.003

Table 3. Tukey post hoc comparisons between *in vitro* chemical treatments and pupa manipulations.
Following a GLM showing a significant interaction between chemical treatment (water or synthetic poison) and pupae manipulation (cocooned, experimentally unpacked or experimentally unpacked and bitten), we performed Tukey post hoc comparisons to determine the influence of each behavioural component. Comparisons to pupae that received complete destructive disinfection (unpacked + poison + biting) are shown in bold. All *P* values are corrected for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.05$).

Post-hoc comparison		**Corrected *P* value**
Cocooned + water	Cocooned + poison	0.26
Cocooned + water	Unpacked + water	0.50
Cocooned + water	Unpacked + poison	0.05
Cocooned + water	Unpacked + water + biting	0.28
Cocooned + water	**Unpacked + poison + biting**	**0.002**
Unpacked + water	Unpacked + poison	0.02
Unpacked + water	Cocooned + poison	0.08
Unpacked + water	Unpacked + water + biting	0.61
Unpacked + water	**Unpacked + poison + biting**	**0.001**
Biting + water	**Unpacked + poison + biting**	**0.001**
Biting + water	Cocooned + poison	0.04
Biting + water	Unpacked + poison	0.01
Cocooned + poison	Unpacked + poison	0.37
Cocooned + poison	**Unpacked + poison + biting**	**0.01**
Unpacked + poison	**Unpacked + poison + biting**	**0.02**

4. Cocoons protect ant pupae against the harmful side effects of chemical disinfection

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Unpublished – manuscript in preparation for submission to *Biology Letters*

Abstract
Many social insects sanitise their nests using self-produced chemicals, symbiont-derived antimicrobials or compounds collected from the environment. In the ant *Lasius neglectus*, workers produce a formic acid rich poison that is applied to nestmates to prevent the infection and transmission of common soil pathogens. Here, we found *L. neglectus* ants also prophylactically treat their nest environment with poison, including chambers containing the brood. Because of the caustic nature of the poison, we hypothesised that the cocoon of the delicate, weakly sclerotized pupae may protect during nest sanitation. Through a series of experiments comparing the survival of cocooned pupae to artificially "nude" ones that we created, we show that pupae are indeed damaged by the poison spraying of ants. However, we find that the cocoon, when present, protects pupae from harm, most likely because of its hydrophobic properties. As the cocoon has been lost in many other species that do not spray poison, this finding suggests that acidic nest sanitation may be selecting for the maintenance of cocoon spinning in *L. neglectus*. Overall, our study demonstrates that collective disease defences could cause damage, or "immunopathology", but counter measures exist to limit negative side effects.

Keywords
nest sanitation; social immunity; disease defence behaviour; cocoons; brood

Author contributions
Conceived and designed study: CDP, SC
Performed experiments: CDP
Analysed data: CDP
Wrote paper: CDP, SC

Experimental work in detail
Acid spraying in nest (Figure 1): CDP (100%)
Protective effect of cocoon (Figure 2): CDP (100%)

4.1 Introduction

Regulating disease defences so that they keep pathogens at bay whilst causing minimal injury to the host is an essential tenant of immunity [7]. However, immunopathology – collateral damaged caused by an immune reaction – can occur and is thought to constrain the evolution of host resistance [156]. Social insects perform behavioural disease defences that function like the immune system of a body to protect the entire colony from harm [17,51]. This social immunity should have also been shaped by selection so that healthy individuals are not damaged by their nestmates performing antiseptic behaviours.

Many social insects utilise antimicrobial compounds that are incorporated into the nest material and applied to brood and workers [136,217]. For example, all Formicine ants produce an acidic (pH ~ 2) poison that is sprayed from an acidopore at the tip of their abdomens [55,136]. When the invasive garden ant, *Lasius neglectus*, encounters pathogen-exposed brood, they use this endogenously produced poison as a chemical disinfectant. Interestingly, it is taken up from the acidopore into the ant's mouths and applied orally during grooming [55]. Combined with the fact that the poison is highly caustic and used to kill other ants [157], this careful oral application suggests that applying the poison directly to individuals has the potential to cause harm.

In many Formicine ants, the developing pupae are covered in silk cocoons that they spin as larvae [251]. The exact function of the cocoon is unclear [149], but it is costly for the larvae to produce [166] and can impede fungal infection [149]. Previous work has established that the cocoon also forms a barrier around the pupae that prevents the ants' poison passing through [56]. If a pupa falls sick, the ants therefore have to remove the cocoon in order to inhibit the infection. Hence, under disease-free conditions, we hypothesised that the pupal cocoon may function to protect healthy pupae from poison use in the nest.

4.2 Results

By keeping *L. neglectus* colonies on pH sensitive paper, we confirmed that they frequently spray poison in their nest in the absence of pathogens (Fig 1; comparison of nests between 3 vs. 48 h: paired *t*-test, $t = -3.81$, df = 6, $P = 0.008$). To determine whether this prophylactic poison use affects the health of the pupae, we compared the survival of pupae experimentally removed from their cocoons ("nude") to that of cocooned pupae, kept with and without ants.

In the presence of ants, we found that nude pupae had significantly reduced survival compared to those still cocooned (Fig 2a; likelihood ratio test (LR) $\chi^2 = 15.43$, df = 1, $P = 0.001$); yet, nude and cocooned pupae kept without ants survived equally well (80 vs. 85 %; LR $\chi^2 = 0.17$, df = 1, $P = 0.68$). We therefore hypothesised that the pupae are suffering from damage caused by poison spraying within the nest. We tested this by keeping nude pupae with acidopore-blocked ants that cannot spray poison or control ants that could.

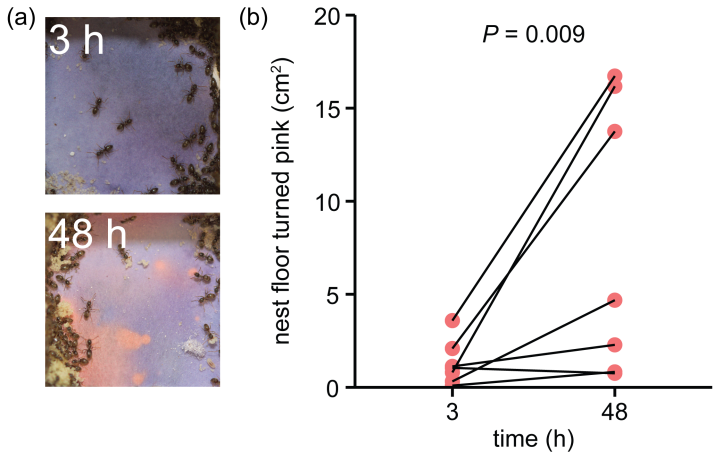

Figure 1. Prophylactic poison spraying in ant nests. (*a*) Representative examples of nests 3 and 48 h after ants were added. Poison spraying (pink areas) visualised by lining nest floor with pH sensitive paper. (*b*) Ants regularly spray poison in their nests over time (paired data with *t*-test *P*-value displayed).

We found that 80% of nude pupae kept with acidopore-blocked ants survived, whereas only 10% made it to adulthood when the tending ants could still spray poison (Fig 2b; LR χ^2 = 11.02, df = 1, P = 0.0001). To test whether the poison is causing this mortality, we treated nude pupae with either synthetic ant poison or water as a control. Indeed, we found that poison spraying significantly reduced the survival of nude pupae, whereas water did not (Fig 2c; LR χ^2 = 19.54, df = 1, P = P = 0.0001).

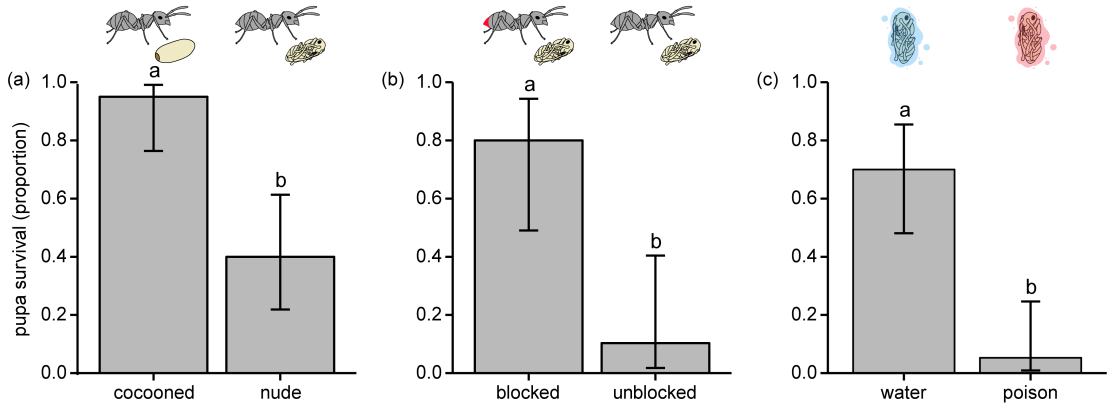

Figure 2. Cocoons protect pupae from ant poison spraying. (*a*) Nude pupae have lower survival in the presence of ants than those cocooned. (*b*) Nude pupae kept with acidopore-blocked ants had higher survival than those kept with unblocked ants still able to spray poison. (*c*) Spraying pupae with synthetic ant poison decrease their survival compared to a water control. Error bars show ± 95% confidence intervals and letters denote significantly different groups (P < 0.05; logistic regressions).

4.3 Discussion

Our results indicate that cocoons protect developing pupae against the prophylactic use of acidic poison within *L. neglectus* nests. During metamorphosis, ant pupae undergo complex, physiological changes and their cuticles become thin and weakly sclerotized [251]. This

probably makes them particularly susceptible to the caustic poison. Poison spraying may be so frequent in ants because, unlike bees and wasps, they keep their brood in piles directly on the soil [251], which can contain high abundances of soil-borne pathogens [48]. Hence, regularly spraying poison over the brood is likely necessary to reduce the probability of infection [217].

Consistent with previous work, we found that the protective effect of the cocoon is due to its ability to block the poison [56]. This is probably because insect silk is made of hydrophobic proteins that form a barrier against aqueous substances [252]. It is tempting to speculate that poison spraying has maintained the need for costly cocoon spinning in the Formicines, especially as most other ant subfamilies lacking this trait also have nude pupae [251]. Curiously, in the fungus-growing ants workers even cover their pupae in fungi [253]. As these ants produce acidic antimicrobials to control microbial growth in the fungus gardens [103], these makeshift "cocoons" may protect the pupae from these chemicals, similar to our results presented here. Yet, within the Formicines, some species have a mix of nude and cocooned pupae, whilst a few lack cocoons completely [251]. Comparative work is therefore needed to assess if, for example, these species have less acidic poison or pupae with tougher cuticles.

Disinfectants are an important component of colony life, having increased in antimicrobial activity with the evolution of social complexity [217,254]. Yet, the costs of producing antimicrobials have so far only focussed on individual-level energetic trade-offs [255]. Our results suggest that the potency of antimicrobials may also be limited by their potential to cause harm within the colony. By having larvae that spin cocoons, *L. neglectus* ants can use their poison without causing damage – or immunopathology – to developing pupae. The absence of this risk should therefore relax constraints on the evolution of the poison's potency. Future studies that investigate the costs of social immunity behaviours will aid in our understanding of their evolution and trade-offs with other life history traits.

4.4 Methods

Lasius neglectus colonies were collected from Seva, Spain, and reared in the laboratory at 23°C with 70% humidity and a day/night cycle of 14/10 h. Pupal age was kept consistent throughout by using pupae with similarly melanised eyes.

We tested if ants spray poison in their nests by taking similarly sized portions of brood, workers and queens (approx. 300 individuals) from stock colonies and placing them into mini-nests (n = 7). Mini-nests consisted of a foraging area (20 x 12 cm) with 25% sucrose solution and minced *Blaptica dubia* provided *ad libitum*, and a covered, chambered nestbox (10 x 3.5 cm; Bock GmbH & Co. KG). The floor of the nestbox was lined with blue pH sensitive paper (Indigo® Instruments) that turns pink on contact with acid. We let the ants settle for 3 h before taking photographs (iPhone 6s) of the nest boxes. After a further 48 h, we photographed them again. The area of the nest floor turned pink was then measured using ImageJ [256].

To determine if pupae lacking cocoons suffer a higher mortality than cocooned pupae, we created nude pupae by experimentally removing their cocoons (as in [56]). Individual nude and cocooned pupae were then kept with a pair of ants in plastered petri dishes (Ø = 35 mm) and *ad libitum* 25% sucrose solution. To ensure that cocoon removal did not increase pupal mortality itself, we kept nude and cocooned pupae under the same conditions, but without

ants. We then recorded the number of nude and cocooned pupae surviving pupation or dying prematurely (n = 20 for all groups).

We investigated the impact of ant poison on nude pupa survival by blocking the acidopore of ants (as in [55]). Briefly, ants were cold immobilised and their acidopore depleted of poison. The gland opening was then sealed with glue and a layer of red paint (Uni Posca). A separate group of ants were cold immobilised but their acidopores were left unblocked. A single acidopore blocked or unblocked ant (n = 10 in both) and nude pupa was kept as above. Ants in both treatments were replaced daily as acidopore blocking is only effective for ~ 24 h. The experiment ended once all pupae eclosed or died.

To confirm that ant poison is toxic to pupae, we kept nude pupae on plastered dishes and sprayed them daily with either water or synthetic ant poison (60% formic acid, 2% acetic acid; developed by Tragust *et al.* [55]). The experiment ended once all pupae eclosed or died.

All statistical analyses were carried out in R 3.3.2. We analysed poison spraying within ant nests using a paired t-test on log-transformed data. Pupal survival was analysed in all experiments with logistic regressions (GLMs with binomial error and logit link function): full and null models were compared to assess the effect of predictors using Likelihood Ratio (LR) tests. Where necessary, we used Tukey post hoc comparisons with Benjamini-Hochburg correction for multiple testing.

Acknowledgements
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5. Co-founding ant queens prevent disease by performing prophylactic undertaking behaviour

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Unpublished – Under review at *BMC Evolutionary Biology*

Abstract
Social insects form densely crowded societies in environments with high pathogen loads, but have evolved collective defences that mitigate the impact of disease. However, colony-founding queens lack this protection and suffer high rates of mortality. The impact of pathogens may be exacerbated in species where queens found colonies together, as healthy individuals may contract pathogens from infectious co-founders. Therefore, we tested whether ant queens avoid founding colonies with pathogen-exposed conspecifics and how they might limit disease transmission from infectious individuals. Using *Lasius niger* queens and a naturally infecting fungal pathogen *Metarhizium brunneum,* we observed that queens were equally likely to found colonies with another pathogen-exposed or sham-treated queen. However, when one queen died, the surviving individual performed biting, burial and removal of the corpse. These undertaking behaviours were performed prophylactically, i.e. targeted equally towards non-infected and infected corpses, as well as carried out before infected corpses became infectious. Biting and burial reduced the risk of the queens contracting and dying from disease from an infectious corpse of a dead co-foundress. We show that co-founding ant queens express undertaking behaviours that, in mature colonies, are performed exclusively by workers. Such infection avoidance behaviours act before the queens can contract the disease and will therefore improve the overall chance of colony founding success in ant queens.

Keywords
host-pathogen interactions; social immunity; disease defence behaviour; entomopathogenic fungus; pleometrosis

Author contributions
Conceived and designed study: CDP, SC
Performed experiments: CDP
Analysed data: CDP
Wrote paper: CDP, SC

Experimental work in detail
Pathogen exposure and colony founding choice (Figure 1): CDP (100%)
Undertaking behaviour in queens (Figures 1-4: CDP (100%).

5.1 Background

Behaviour that decreases the probability of an individual acquiring pathogens should confer fitness advantages and be selected for over time [257]. This is because mounting an immune response post-infection can have a severe impact on an animal's future reproduction and survival, whilst behavioural mechanisms can negate these costs by preventing infection altogether [258–261]. Most often, these behaviours are the avoidance of contagious conspecifics or areas they have contaminated [20]. For example, animals avoid sheltering, interacting and mating with infectious counterparts [23–25], and in humans, disgust at disease-associated stimuli is thought to be adaptive as it should reduce pathogen exposure [26,27]. Behavioural responses that minimise infection risk are therefore considered an important but less well studied component of a host's disease defence repertoire [7,262].

In social insects (ants, termites and some bees and wasps) workers perform collective behaviours, such as grooming, which reduce the *per capita* risk of infection and result in colony-level disease protection, known as social immunity [17]. However, daughter queens often lack this protection as they typically leave the parental nest and found new colonies without the assistance of workers [17]. The rate of mortality for founding queens is therefore high and many die as a result of disease [42,68,79,80,263]. In some ant species queens found new colonies with other, usually unrelated queens, known as co-founding or pleometrosis [76]. Although co-founding can improve queen survival [73,80], we suggest it may also increase the queens' risk of disease if co-foundresses fall sick and become infectious. For example, fungus-infected ants can release millions of new infectious spores after death, which significantly reduce the survival of other colony members [202,203]. Furthermore, even non-diseased corpses have negative impacts on worker and brood survival if they are not removed from the nest [138]. We therefore suggest that dead and/or infectious co-foundresses could affect the colony founding success of surviving queens. Subsequently, we might expect selection acting on queens to produce behaviours that reduce this risk.

Ant queens can assess the quality (e.g. the size and condition) of their conspecifics and this affects who they co-found with in the laboratory [72]. As social insect queens can also detect pathogens [74,134,264], they may therefore avoid co-founding with pathogen-contaminated queens to reduce their own infection risk. However, the decision to co-found is influenced by several factors, including nest site availability and the danger of desiccation, which could supersede co-founder choosiness [264]. In addition, ant queens may perform behaviours that prevent pathogen transmission from infected co-foundresses, similar to colony founding termites, which have been reported to groom and bury freeze-killed co-founders, thereby reducing subsequent saprophytic microbial growth on their corpses [265]. However, it remains unclear if these corpse-induced responses, known generally as "undertaking behaviours" [266], actually affect disease transmission, as they are expressed immediately following death, whilst the infectious potential of the corpse may only become evident later.

Here, we therefore investigated if and how queens of the black garden ant, *Lasius niger,* are able to reduce their risk of contracting disease from co-foundresses. In *L. niger*, virgin queens leave the parental nest to engage in mating flights. Afterwards, they search for and establish a nest, with co-founding occurring in about 18% of cases (usually in pairs) [66]. During colony founding, *L. niger* queens are naturally infected by several fungal pathogens, including the generalist insect pathogen *Metarhizium brunneum* (CD Pull, unpublished data). These pathogens can be found in abundances of up to 5000 infectious conidiospores/g of soil [48] and insects acquire infections when conidiospores attach to, and penetrate, their cuticle. During the ensuing non-infectious incubation period, the fungus proliferates and eventually

causes host death, before sporulating and producing a new generation of infectious conidiospores on the corpse [84].

Using this host-pathogen system, we set up a choice experiment to first investigate how pathogen exposure affects the co-founding decision of queens. We tested if queens avoid co-founding less with pathogen-exposed individuals, compared to sham-treated control queens. Secondly, we studied the behaviour of queens following the death of a co-foundress. We compared how and when queens reacted to both infected and non-infected corpses, and predicted that the queens' response should differ based on the risk of infection. We then examined whether the behaviours performed by the queens prevent the pathogen from becoming transmissible and infecting the surviving queen.

5.2 Results

5.2.1 Pathogen exposure and colony co-founding choice

In our first experiment, we set out to determine how pathogen contamination affects the co-founding decision of queens, from both the perspective of a queen already in the nest and those that may join her. We introduced pathogen-exposed or sham-treated queens to an experimental setup where they could choose to start a nest alone, or with a pathogen-exposed or sham-treated queen already residing in the nest, using a full factorial design. We observed no effect of pathogen-exposure on the likelihood that queens co-found colonies within a 72-hour observation period (Figure 1; overall generalised linear mixed model [GLMM] comprising queen treatment, time and their interaction, $n = 20$ per treatment group, likelihood ratio test (LR) $\chi^2 = 4.95$, df = 7, $P = 0.7$). On average, 65% of queens across the queen combinations decided to co-found, showing that pathogen exposure does not affect the colony co-founding choice of either the residing or the introduced queen.

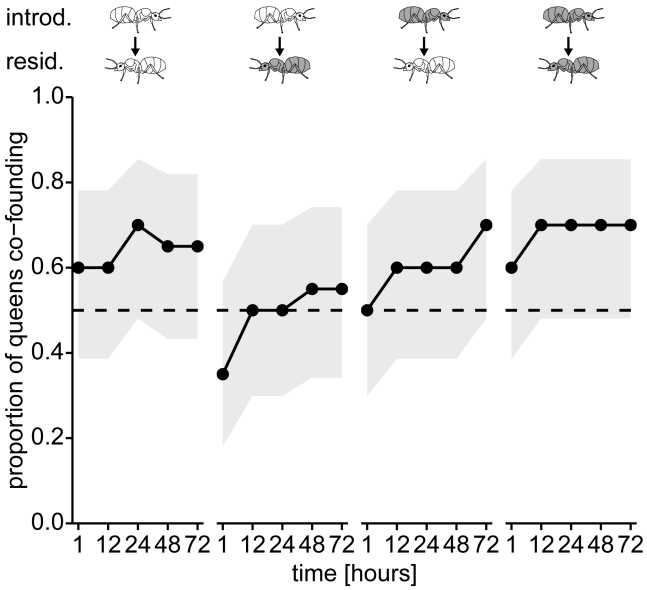

Figure 1. Queen co-founding is independent of pathogen exposure. Introduced queens that were either pathogen-exposed (grey queens) or had received a sham treatment (white queens) were given the choice of founding a colony alone or with a residing queen already present in the nest, which had either been pathogen-exposed (grey queens) or sham-treated (white queens). There was no significant effect of the queen combinations and time on the co-founding decision of queens, revealing that pathogen exposure of neither the residing nor the introduced queen affects the colony co-founding choice (points ± grey shading represent proportions ± 95% confidence intervals for each time point; 0.5 dashed line added for visual interpretation; GLMM model non-significant).

5.2.2 Queen undertaking behaviour

In a second experiment, we investigated the response of untreated queens to corpses by pairing them with either a pathogen-exposed or sham-treated queen. When a pathogen-exposed queen died of infection (median survival time = 6 days), we freeze-killed a sham-exposed queen to test if the untreated queens react differently to infected and non-infected corpses. Queens were kept in either closed nests (a single chamber) or open nests (single chamber with an exit hole that opens into an arena) that contained dried plaster particles as nesting material. We observed the queens performing three undertaking behaviours towards the corpses of both infected and non-infected corpses. In closed nests, 74% of the queens dismantled the corpses by biting them to remove the limbs and break up the body segments and, in 62% of the cases, queens buried the corpses with the plaster particles from the nest. These behaviours were performed equally towards infected and non-infected corpses, with no significant differences in the occurrence of biting (Figure 2a; infected: 16/23, non-infected: 19/24; GLM, LR $\chi^2 = 0.57$, df = 1, $P = 0.45$) or burial observed (Figure 2b; infected: 15/23, non-infected: 14/24; GLM, LR $\chi^2 = 0.24$, df = 1, $P = 0.63$). These behaviours typically occurred shortly after the death of the co-foundress (median day: biting = 1, burial = 2) and there was no difference in onset regardless of corpse type (biting: infected $n = 16$, non-infected $n = 17$; Mann-Whitney U test, $U = 112$, $P = 0.38$; burial: infected $n = 11$, non-infected $n = 13$; Mann-Whitney U test, $U = 64$, $P = 0.68$). In open nests, the predominant behaviour, occurring in 78% of the cases, was the removal of the corpse from the nest

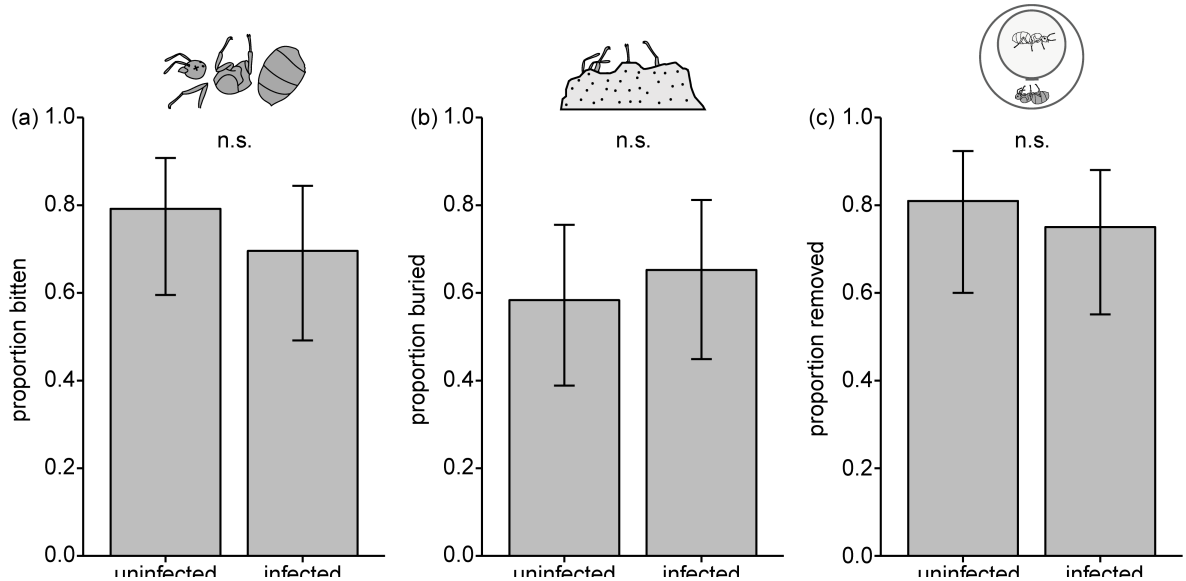

Figure 2. Queens perform prophylactic undertaking behaviours towards co-foundress corpses. Queens encountering non-infected and infected corpses performed several behaviours, namely corpse (a) biting and (b) burying or (c) removal. These behaviours were performed equally towards both infected and non-infected corpses (error bars show ± 95% CI; n.s. = non-significant GLM result at $\alpha = 0.05$).

chamber into the external arena, an undertaking behaviour termed necrophoresis [266]. Again, necrophoresis was performed equally towards both infected and non-infected corpses (Figure 2c; infected: 18/24, non-infected: 17/21; GLM, LR $\chi^2 = 0.23$, df = 1, $P = 0.63$), which were also removed from the nest at similar times after co-foundress death (median day: 1; infected $n = 17$, non-infected $n = 17$; Mann-Whitney U test: $U = 152$, $P = 0.81$). However, in cases where corpses were left inside the nest (19% of the control and 25% of the pathogen group), queens also performed biting and burial, as in the closed nests (infected: biting occurrence in 1/6 of the replicates, burial in 2/6; non-infected: biting in 2/4, burial in 3/4).

5.2.3 Fungal outgrowth and onset of undertaking behaviours

In 96% of cases (45/47), the corpses of pathogen-exposed queens sporulated and produced new infectious conidiospores, meaning that the behaviours performed by the queens seem generally unable to prevent fungal reproduction. However, queens typically performed undertaking behaviours before the fungus became infectious (% of nests where behaviours occurred before sporulation: biting = 100%; burial = 85%, removal = 82%). Biting, burial and removal all occurred significantly earlier than sporulation (Figure 3; biting: $n = 15$, Wilcoxon signed-rank test, $Z = -3.43$, $P = 0.002$; burial: $n = 13$, Wilcoxon signed-rank test, $Z = -2.92$, $P = 0.005$; removal: $n = 17$, Wilcoxon signed-rank test, $Z = -2.6$, $P = 0.01$).

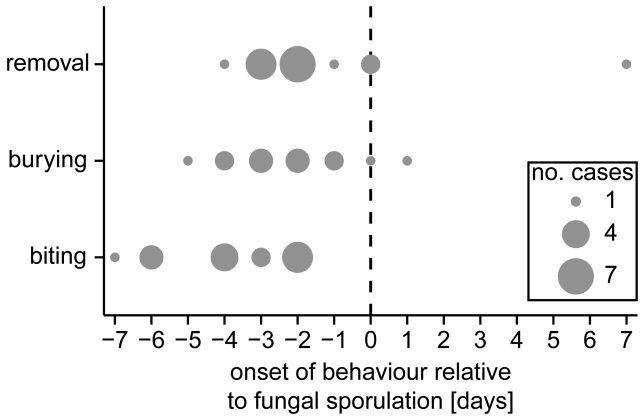

Figure 3. Undertaking behaviours precede corpse sporulation. Queens performed biting, burial and removal before infected corpses sporulated and became infectious. The day behaviours were performed are displayed as points and are relative to fungal sporulation (time point 0; dashed black line), meaning that negative points = before sporulation, and positive = after. The size of the points indicates the number of cases that occurred per day.

5.2.4 Effect of undertaking behaviours on disease transmission

There was no mortality in untreated queens presented with non-infected corpses within the 30 days of the experiment. In contrast, 45% of queens (21/47) in contact with infected corpses died and 95% (20/21) of these sporulated, indicating that the cause of queen death was a *Metarhizium* infection contracted from the corpse. However, we found that the undertaking behaviours the queens performed significantly affected their survival. In cases where infected corpses were inside the nest (closed nests and open nests where removal was not performed), both biting (Figure 4a; bitten: 12/17, unbitten: 3/12; GLM, LR $\chi^2 = 6.1$, df = 1, $P = 0.02$) and burial (Figure 4b; buried: 12/17, unburied: 3/12; GLM, LR $\chi^2 = 6.1$, df = 1, $P = 0.02$) caused a significant reduction in the number of the queens dying. Additionally, the removal of infected corpses reduced chances of a queen dying, yet this difference was non-significant (Figure 4c; removed: 11/18, unremoved: 2/6; GLM, LR $\chi^2 = 1.41$, df = 1, $P = 0.24$).

5.3 Discussion

Our results reveal that ant queens do not avoid founding nests with pathogen-exposed conspecifics (Figure 1). But, if a co-foundress dies, queens will bite and bury the corpse or remove it from the nest (Figure 2). These undertaking behaviours occurred shortly after death and, in the pathogen group, before corpses became infectious (Figure 3). Importantly, we found that the biting and burying of corpses improved queen survival (Figure 4).

Although ant queens risk contracting infections from pathogen-exposed co-foundresses, we show that ant queens do not avoid founding colonies with contaminated individuals. This is most likely because social insect queens have to rapidly find and dig a nest before they succumb to desiccation or predation, and it has been postulated that these risks should therefore outweigh spending time assessing co-founders [264]. Moreover, the patchiness of suitable nest sites and the resultant overcrowding likely forces queens to share, regardless of co-foundress quality [72]. As co-founding can improve queen survival, this could explain their general tendency towards founding colonies together rather than alone [73,76,80].

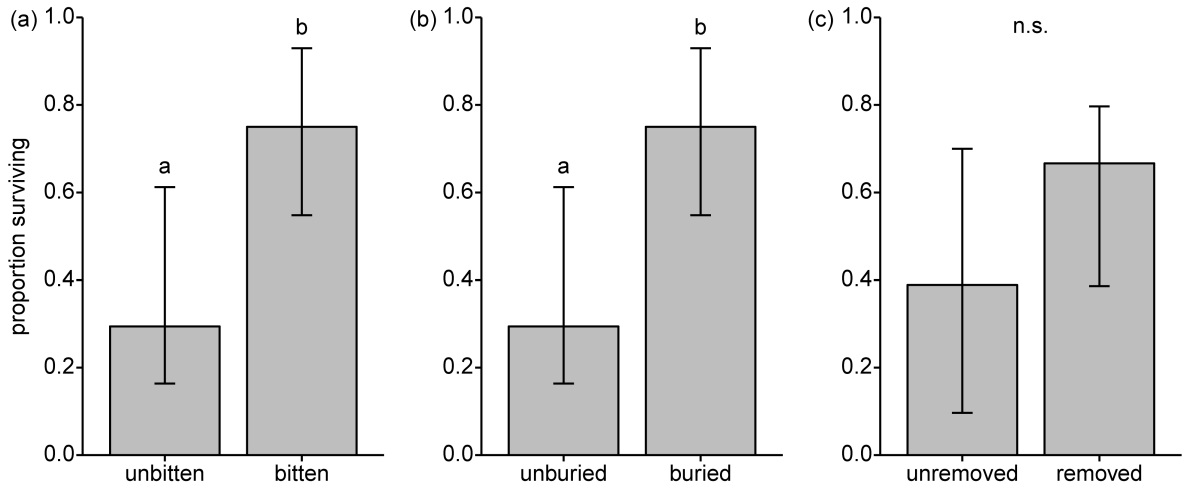

Figure 4. Biting and burial of co-foundress corpses improve queen survival. The number of queens surviving following the death of a co-foundress due to infection, was significantly increased when they performed (a) biting and (b) burial, whereas there was no significant effect for (c) removal (error bars show ± 95% CIs given; letters denote significant GLM results at $\alpha = 0.05$; n.s. = non-significant).

When a co-foundress died, we observed the surviving ant queens performing undertaking behaviours that have so far only been described to be performed by workers [266]. In general, when nests were open, queens performed necrophoresis, whilst when they were closed, biting and burial was performed instead. Hence, our results reveal that although ant queens in mature colonies focus on reproduction, they can perform sanitary behaviours during the early, workerless stages of their lifecycle. Interestingly, we observed queens performing undertaking behaviours prophylactically towards both non-infected and infected corpses, which is in line with studies on workers showing that undertaking and self-removal occur in the absence of infection and before disease symptoms appear [266]. Importantly, the onset of these behaviours also preceded the fungus sporulating and becoming infectious. Acting prophylactically may be important as both infected and non-infected corpses can impact the survival of nestmates, e.g. due to the post-mortem growth of infectious and saprophytic microorganisms [138,142,202,203]. Moreover, acting early may maximise the likelihood that disease transmission is prevented, as pathogenic fungi usually take 1-2 days to sporulate [56,142]. The diverse repertoire of undertaking behaviours we observed in queens is probably important given that, at certain times of the year, necrophoresis could be difficult to perform because the ground is too hard for the queens to dig an additional chamber to place the corpse. Additionally, excessive digging can increase queen mortality, presumably because they exhaust the body reserves that they store for sustenance and brood-rearing [267], and it may expose them to further pathogens in the soil. Hence, in the wild, the type of undertaking behaviour performed may be influenced by several factors.

Forty eight per cent of queens that founded colonies with pathogen-exposed individuals contracted infections and died. This mortality could have resulted from cross-contamination that occurred after pathogen-exposed queens were introduced to the nest, i.e. before the fungal conidiospores had adhered to their cuticles and could have been transferred to the untreated queen [53,268]. Such cross-contamination could occur via contaminated nest surfaces, but is most likely to arise during allogrooming, which *L. niger* queens do not perform [74]. Moreover, such infections are normally low-level and thus non-lethal [53]. In addition, all untreated queens in cases where the pathogen-exposed queens survived (11/58 nests) did not contract lethal infections, suggesting that untreated queens in the other nests

most likely contracted infections from the sporulating, infectious corpses. Indeed, they all died only after the sporulation of the co-foundress corpse.

Given that the queens perform undertaking behaviours on their own, it may not be surprising that they cannot prevent sporulation, which in mature colonies requires the concerted action of multiple workers [56]. Despite this limitation, however, queens that performed biting and burial had improved survival. We noted from visual inspections that corpses bitten and buried by the queens supported less conidiospore growth than those that were only removed from the nest or single queens kept in isolation. It has been suggested that biting can desiccate corpses and create unfavourable conditions for the pathogen [17]. In our experiment, humidity was optimal for fungal growth throughout, so that biting the corpses into smaller pieces might give the additional benefit of limiting the amount of nutrients available for the fungus and result in a reduction in the total amount of new infectious conidiospores produced from corpses. As the probability of mortality from a *Metarhizium* infection is directly linked to exposure dose [89,269], biting may therefore improve the survival of the co-founding queen by reducing how many infectious propagules she is confronted with from the sporulating corpse.

Corpses that have been dismantled through biting may also be buried more efficiently and both behaviours were usually performed together. Burial is an effective undertaking behaviour as it physically separates the infectious corpse from susceptible insects [109,265,270]. Thus, queens that perform this behaviour should have a lower risk of contracting an infection than queens who did not. Whether buried corpses will be excavated as the colony grows is unknown. However, areas of the nest containing buried corpses are avoided by termites [109] and the infectivity of *Metarhizium* conidiospores decreases with time [271,272], making it less likely that co-foundress corpses cause infections after the first workers emerge.

In contrast to biting and burial, necrophoresis did not significantly enhance queen survival. This is surprising because, as in mature ant [138,139,142,212] and honeybee [143] colonies, this behaviour isolates the corpse and should prevent disease transmission. The absence of a statistically significant difference, despite an improvement in the survival in the queens performing the behaviour, could have been driven by the low number of queens not performing the behaviour (6/24), which likely reduced our statistical power. However, it is also possible that the queens still interacted with the corpses after removal. Indeed, both ant [134] and termite queens [264] appear to be attracted to conidiospores in their environment and removed corpses were typically left unbitten and unburied, so contact with the corpses would lead to disease transmission and thus infection.

5.4 Conclusion

In this study, we have characterised the undertaking behaviours of colony founding ant queens. Whilst co-founding queens neither avoid pathogen-exposed individuals (this study) nor perform any sanitary care towards them [74], here we have shown that they are indeed able to perform undertaking behaviours, which are typically considered worker-tasks in mature colonies. In the absence of workers, the queens therefore act to prophylactically protect themselves from disease. Although several studies have demonstrated the immunological capabilities of founding ant queens [74,164,273–275], avoiding infection may be particularly important because they are ‘closed systems’, surviving solely on the breakdown of fat and muscle reserves until the first workers emerge and begin foraging

[70,78]. Queens must therefore balance their limited resources and investment into immunity can cause trade offs with other life history traits [74,164]. For example, queens exposed to *Metarhizium* can survive infection by resisting the pathogen, however, they subsequently produce fewer workers than healthy individuals [74]. Smaller colonies are at a competitive disadvantage and are more likely to fail before new sexual offspring are produced [76]. Although protected by workers in mature colonies, social insect queens, like other animal taxa [20], should therefore be under selection to evolve both immunological [164,258,273–275] and behavioural (this study, [265]) adaptations against disease, in order to survive the risky colony founding stage.

5.5 Methods

Queen collection and maintenance
We collected hundreds of mated queens in July 2013 and 2014 from the IST Austria campus, Klosterneuburg, Austria. Queens were returned to the laboratory in plastic boxes containing damp tissue paper until they were used in experiments. No food was provided to queens in any of the experiments as they survive solely on the breakdown of muscle and fat reserves during colony founding [70].

Fungal pathogen
We used the species *M. brunneum* (strain KVL-03-143), collected from and grown on sabaroud dextrose agar plates before each experiment. Conidiospores were suspended in autoclaved 0.05% Triton-X and their viability confirmed by plating out 100µl of the conidiospore suspension onto sabouraud dextrose agar plates and checking the number of conidiospores germinating after 18 hours (always > 90%).

Queen pathogen exposure
Queens were exposed to the fungal suspension or autoclaved Triton-X as a sham- exposure, by gently restraining them with soft forceps and pipetting 0.5µl of the fungal suspension or Triton-X onto their thorax. Queens were then placed onto filter paper to remove excess liquid and allowed to dry alone for several minutes before being added to experiments. For all experiments, we applied a droplet of 0.5 µl (2×10^7 conidiospores ml^{-1}), which causes high mortality in queens (30/35 queens) kept alone for 30 days (median survival time = 6 days).

Experiment 1: Effect of pathogen exposure on colony co-founding choice
All queens were individually colour-marked (paint "Uni Posca") on one of their abdominal segments to differentiate the pathogen-exposed and sham-treated queens. We set up plastic boxes (10 x 3.5 cm; Bock GmbH & Co. KG) comprising three equally sized chambers and a transparent lid. The middle chamber was uncovered and had no substrate, whereas the two chambers on either side were covered in red transparent foil (to reduce light entering the chamber whilst allowing observations of the ants) and had a damp plaster substrate. A hole (5 mm Ø) in the walls of the middle chamber connected it to the two adjacent chambers, allowing the ant queens to move freely between them.

Into one of the plastered and darkened chambers, we placed either a sham-treated or pathogen-exposed queen, which we termed the “residing queen”. After allowing her time to settle (1 hour), we then introduced a second queen to the middle chamber, which was either sham-treated or pathogen-exposed, and termed the “introduced queen”. We varied which of the two darkened chambers the first queen was placed into in case there was a directional bias towards one of the chambers.

Thus, we had four experimental groups (i) a sham-treated queen introduced to a nest with a sham-treated residing queen (ii) a sham-treated queen introduced to a nest with a pathogen-exposed residing queen (iii) a pathogen-exposed queen introduced to a nest with a residing sham-treated queen and (iv) a pathogen-exposed queen introduced to a nest with a residing pathogen-exposed queen (n = 20 in all cases). Following the introduction of the second queen, we observed the locations of queens after 1, 12, 24, 48 and 72 hours. We stopped observations at 72 hours as ~ 75% of queens had produced eggs and queens started dying from the fungal exposure after this point. The experiment was run at 23°C and 70% humidity, under continuous light.

Experiment 2: Queen behaviour towards co-foundress corpses and disease transmission
We placed single, unpainted and untreated queens into petri dishes (Ø = 3.5 cm) filled with damp plaster that contained a rectangular cavity measuring 1 cm x 3.5 cm, to mimic the small chambers queen's construct when founding a colony. Each chamber contained 1g of loose plaster particles as a nest material. The lids of the dishes were covered with red transparent foil to keep the chamber darkened. We termed these dishes "closed nests". Half of the dishes were then placed into a second, larger dish (Ø = 9 cm) with a plaster substrate. A small hole (Ø = 5 mm) in the side of the small dish allowed the queen access to this external arena, to create an "open nest". We then added a second, paint-marked queen (allowing us to distinguish her from the untreated queen) to each dish, which was either sham-treated or pathogen-exposed.

We monitored the survival of pathogen-exposed queens on a daily basis and noted when she died. So that we could compare the behaviours of naive queens towards infected and non-infected corpses, we removed and froze a sham-treated queen to create non-infected corpses. These queens were frozen, on the same day that a pathogen-exposed queen died, for 5 min at – 80°C, before being added back to the dish with the surviving queen. The pathogen-exposed queens died in the majority of nests (47 out of a total of 58 that we set up) and those where she did not were not included in the analysis. Overall, we therefore had four treatment groups: (i) untreated queens in closed nests with the corpse of a pathogen-exposed queen (n = 23); (ii) untreated queens in closed nests with the corpse of a sham-treated queen (n = 24); (iii) untreated queens in open nests and the corpse of a pathogen-exposed queen (n = 24); (iv) untreated queens in open nests and the corpse of a sham-treated queen (n = 21).

On a daily basis, we recorded the behavioural response of untreated queens to corpses, as well as when sporulation occurred, through visual inspection. In addition, we recorded the survival of the untreated queens and, when they died, if sporulation occurred on their corpse. In a few cases, the exact timing of the occurrence of the behaviour (2/36 for biting, 5/31 for burial, 1/35 for removal), or sporulation (1/45) was missed. Exact sample sizes per test are provided in the results section. The experiment was run at 23°C and 70% humidity, under a 12 hour light:dark schedule. The duration of the experiment, from pathogen exposure to the final inspection for fungal growth, was 30 days.

Data analysis
All statistical data analysis was carried out using R version 3.3.2 [230]. We analysed the colony co-founding choice of queens using a generalised linear mixed model (GLMM; 'lme4' R package [276]), including chamber choice as a logistic response and experimental group, time (z-transformed) and their interaction as predictors. To control for the repeated observation of the same replicate, a random intercept was included for each replicate, and

their individual differences over time were explicitly modelled by including random slopes for each individual. General linear models (GLMs) with binomial error terms and logit-link functions were used to compare the behaviour of queens towards infected and non-infected corpses, including the presence/absence of the behaviour (biting, burial or removal) as the response and the type of corpse (infected or non-infected) as the predictor. Mann-Whitney *U* tests were used to test for differences between the day of onset of undertaking behaviours between infected and non-infected corpses. Wilcoxon signed-rank tests were used to compare the days that the undertaking behaviours and fungal sporulation occurred, and to control for multiple testing, we corrected the resulting *P*-values using the Benjamini-Hochberg procedure to protect against a false discovery rate of 0.05% [232]. Adjusted p values are reported. The survival of queens performing different behaviours was analysed using GLMs with binomial error terms and logit-link functions that included mortality of the untreated queen as the response and the presence/absence of the behaviour (biting, burial or removal) as the predictor. Again, we controlled for multiple testing by correcting the *P* values from these models using the Benjamini-Hochberg procedure [232]. We ensured all data fit the assumptions of the models (i.e. variance inflation, Cook's distance, dffits and leverage) and overall model significance, plus the effect of predictors, were tested by comparing full models to nested null and reduced models, respectively, using likelihood ratio tests. All graphs were made using the 'ggplot2' R package [241].

6. Ontogeny of immunity in incipient ant colonies

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Prospective manuscript – Research in progress.

Abstract
Investment into immunity should be a flexible trait that is adjusted to reflect the risk of disease. This is because, as organisms grow and develop, their risk of contracting infections is likely to change, in addition to their ability to invest into immunity. Ant colonies go through several discrete stages of development: a single queen phase where queens must balance investment into immunity and fecundity, an ergonomic phase where the colony is growing but susceptible to perturbations, and stable reproductive stage were new sexual offspring are reproduced. During each of these stages, the risk of disease and the ability to invest into immunity will differ. For example, as colonies grow larger, the risk of disease transmission also increases. At the same time, however, colonies begin performing collective disease defences that can offset this risk. In this study, we investigated how investment into immunocompetence (measured using a sensitive antifungal activity assay) changes, from the solitary queen phase, to colonies with several hundred workers (a time span of one year and two months). We find that investment into immunity is a dynamic process in queens, initially increasing and staying high for the solitary phase, but then fluctuating once a colony has workers. We suggest that this is likely due to the reproductive status of the queen. In contrast, the immunocompetence of workers is considerably higher in colonies equal to or older than one year than in the first workers produced. Hence, despite a widespread array of sophisticated collective defences, investment into physiological immunity seems to remain an important aspect of disease defence in social insects.

Keywords
host-pathogen interactions; disease; social insects; antifungal defence; colony founding; personal and social immunity

Author contributions
Conceived and designed study: CDP, SC
Performed experiments: CDP, EF
Analysed data: CDP
Wrote paper: CDP, SC

Experimental work in detail
Queen collection, weighing, colony maintenance, brood counts (Figure 1): CDP (100%)
Fungal identification: EF
Antifungal assay and analysis (Figures 2-4): CDP (20%), EF

6.1 Background

Organisms should invest into immune defence in order to protect themselves from pathogens [7]. Yet, because immune defences are often costly to maintain, investment into immunity itself can cause trade-offs with fitness related proxies, such as fecundity [259–261]. Hence, organisms should be under selection to balance investment into immunity so that an optimal solution is reached where fitness is maximised [277].

As pathogen exposure and the risk of infection are likely to change as an organisms grows and develops, the level of investment into immunity should be a plastic trait [278–280]. Furthermore, investment into immunity may not always be possible, if, for example, an organism is facing additional stressors, or other life history traits take precedence, such as reproduction [281]. Organisms may therefore transiently adjust their investment into immunity so that disease defences are up or down-regulated, according to the prevailing conditions an organism experiences.

In ants and other eusocial insects, colonies go through several discrete periods of development [42] and drastic seasonal fluctuations in colony size are observed [282]. Colonies are typically founded by single queens who must mate, establish a nest and then use up all their energy reserves in order to raise the first workers [70]. Once workers emerge colonies enter an ergonomic growth phase where the size of colony increases exponentially. During this period the initially limited number of workers makes colonies vulnerable to perturbations, which include intense intraspecific competition with other incipient nests, disease and, in perennial species, hibernation [283]. However, nests that reach a critical size seem more able to buffer such stressors. Consequently, mature social insects colonies often have a reduced extrinsic mortality and can survive many years [172,283]. Between and within each of these different stages, the need for investment into immunity is likely to change. On the one hand, as a colony grows the risk of epizootic will intensity, due to the increasing density of hosts and intimate interactions between them, which encourages pathogen transmission [1,4,8,17]. On the other, social insects express social immunity – a colony-level reduction in the risk of disease – achieved through the cooperatively performed disease defences of workers in conjunction with the social organisation of colonies [17,43,45,122]. Hence, it is difficult to predict how immunity will change with colony growth, but exploring immune investment as colonies develop may help shed light on the relative importance of, and interplay between, social disease defences and immunological ones [284].

Studies of immune investment in social insects have found that the immune activity of founding queens is increased after mating [273,274] and trades-off with sperm storage [164]. In addition, the immunocompetence of workers has been compared to other castes, generally finding that workers have higher immune activity than unmated alates [275,285,286]. In particular, the males seem to invest very little into immune defence, which can be explained by their short lifespans (males of most species die after mating) and high investment into reproduction [286,287]. However, few studies have investigated how immune investment changes with the colony growth and development, where the relative investment into immunity of the different castes is likely to change, e.g. due to the emergence of social immunity [284]. In bumblebees, one study found that workers seem to invest more into immunity as colonies mature [279] and a similar pattern has been observed in fungus-

growing ants [288]. However, neither of these studies examined the immunocompetence of the queen and how her immune investment changes over time.

Here, we aimed to address this gap in our knowledge by performing a longitudinal analysis of constitutive immunocompetence (measured via an antifungal assay) in incipient ant colonies, covering the solitary queen phase up to colonies in the exponential growth phase that contained several hundred workers. To that end, we collected founding queens of the black garden ant *Lasius niger* and allowed them to establish colonies in the laboratory. This species exhibits claustral (non-foraging) colony founding, where nests are initially "closed systems" and queens have to support both themselves and the first brood by metabolising their fat and muscle [70]. Therefore, a balance must be struck between their immune response and fecundity: if a queen invests too much into immunity, she may fail to produce workers fast enough before her resources run out [80], or too few to compete with other incipient colonies [66,73,76]. Yet, if a queen invests too heavily into fecundity, she may leave herself more vulnerable to disease. Conversely, this trade-off may be reduced or eliminated entirely in queens of larger colonies, as the workers can protect them from disease [17]. Furthermore, the immunocompetence of the workers may also change as a colony grows and develops. For example, the first workers – known as nanitics – may be more susceptible to disease than older colony workers because the queens had limited resources to raise them [283,289]. Still, because the first workers are essential for the colony to succeed [76,283], the nanitics could conceivably be more resistant to disease than mature colony workers, as their loss cannot be tolerated [83,284].

6.2 Methods

Queen collection and colony set up
We collected mated (dealated) *Lasius niger* queens from the IST Austria campus during a mating fight in July 2013. Queens were retuned to the laboratory, weighed (using a microbalance; Mettler Toledo) and placed into individual, clear plastic pots (H: 3cm, Ø = 2 cm), which had a substrate of plaster and perforated lid. The pots containing the queens were kept in a climate-controlled chamber at 23ºC, 70% humidity and a 12 h day:light cycle. In addition, the plaster was watered on a weekly basis. As *L. niger* queens are claustral founders (i.e. they do not forage), queens were not fed or provided with sucrose solution during this period. Once the first workers emerged, a hole (Ø = 5mm) was made in the lid of the pot containing the queen and her offspring, and the pot was placed on its side within a larger, cylindrical container (H = 5 cm, Ø = 9cm), which acted as a foraging area. The base of the container was plastered and a gauze-covered opening in the lid (Ø = 2cm) provided airflow. Minced cockroach (*Blaptica dubia*) and a cotton-plugged tube of 25% sucrose solution were added to the container and replaced weekly. To mimic natural conditions, all colonies were moved into hibernation 109 d after the start of the experiment, at 5ºC and 80% humidity, for 150 d (November-April). After which, the colonies were returned to the climate chamber at 23ºC and 70% humidity.

Sampling and colony counts
To study how immunity changes over time, we collected samples 24 hours after the queens' mating flight, 1 week after their first eggs, 1 week after their first pupae, 1 week after their first workers, on the last day of hibernation, 1 year after their mating flight and 1 year after their first workers (total duration of the experiment: 1 year and 2 months). At each time point, we removed, weighed and stored randomly chosen queens (determined using a random

number generator) at – 80 ºC for use in subsequent assays (n = 12 queens per time point for antifungal assay; see below). Using a microscope and hair pick, we counted all the brood present in the colonies of the frozen queens and, if workers were present, stored a subsample (~ 60 workers per colony) at –80 ºC for use in assays.

Dry weight of workers

Since the size of workers was likely to increase as colonies grow [282], we needed to determine the dry weight of workers in order to correct inhibition values. To that end, ten workers per time point (where workers were present) were haphazardly removed from – 80ºC storage and placed into glass vials. The vials were then dried at 60ºC for 24 h. The dry weights of worker pools were then measured using a microbalance.

Fungal pathogen

For the antifungal assay we used the common soil pathogen *Metarhizium brunneum*. The strain used was isolated and cultured from a naturally infected *L. niger* queen that we collected during the mating flight on the campus of IST Austria, which died and showed fungal outgrowth in the laboratory shortly after. To confirm strain identity we sequenced the eukaryotic translation elongation factor 1 alpha gene. Fungal DNA was extracted using Qiagen DNeasy Blood & Tissue Kit according to manufacturer's recommendations, with an elution volume of 50µl. The PCR reaction mix comprised the following reagents: 5 µl 5x MyTaq Red Buffer (Bioline), 1.25 U MyTaq DNA polymerase (Bioline), 10 pmol each of EF1T and EF2T primer, 20 ng DNA and 15.75 µl of RNase free water (Sigma) in a final reaction volume of 25 µl. The primer sequences and cycling conditions were taken from in Bischoff et al 2006 [290]. For PCR clean-up, 8.75 µl of RNase free water (Sigma), 1 U of FastAP™ Thermosensitive Alkaline Phosphatase or Shrimp Alkaline Phosphatase (Fermentas) and 5 U of Exonuclease I (Fermentas) were added to 20 µl of PCR product. The mixture was incubated for 15 min at 37°C, followed by 15 min at 85°C. Products were sequenced by LGC genomics. Sequences were analysed using CLC Main Workbench v. 7.7.1 (Qiagen).

Conidiospores of *M. brunneum* were plated from long-term storage (–80 ºC in glycerol [90]) onto Sabouraud dextrose (SD) agar plates. Fully sporulating plates were harvested in autoclaved 0.05% Triton X-100 and the resulting suspension diluted to the desired concentration (see 'Antifungal activity assay' below). We tested the viability of the conidiospore suspension before running the antifungal assays by plating it onto SD plates, and counting the number of conidiospores that germinated (always > 95%).

Antifungal activity assay

We measured the immunocompetence of queens using a sensitive antifungal activity assay (as in [53]). Individual queens were placed into Eppendorf tubes, snap frozen in liquid nitrogen and crushed using a sterile pestle. 200 µl of PBS buffer was added and the tube briefly vortexed. We then placed the tubes in a centrifuge for 5 min at 3000 cf and 4 ºC. 70 µl of the supernatant was added to a new tube and stored at – 20 ºC for use in the assay.

Before running the antifungal assay, we first determined the necessary ratio of pathogen, ant sample, and buffer to achieve a linear fungal growth curve, in which the antimicrobial activity of the queen sample could be detected. Based on these results, we added 43 µl of SD broth, 5 µl of *M. brunneum* conidiospore suspension (10^9 conidiospores ml^{-1}) and 2 µl of the queen sample to a 96 half well plate (Greiner, clear). In addition to the biological replicates,

we included fungal growth controls (45 µl SD broth and 5 µl conidiospore suspension) and negative controls (45 µl SD broth and 5 µl PBS buffer).

To measure fungal growth inhibition, we added the well plate to a spectrophotometer (SpectraMax M2e, Molecular Devices). We set the spectrophotometer to automatically mix the plate for 20 s before absorbance was measured (wavelength: 595 nm) and recorded absorbance 0 and 27 h after adding the samples to the plate, between which the plate was gently shaken (Vortex Genie) at 23 ºC. At both time points the absorbance of each sample was measured thrice and the mean value taken for statistical analysis.

To measure the fungal growth inhibition of workers, we pooled 5 ants and added them to Eppendorf tubes, which we then treated as above. For the antifungal assay, we used a lower concentration of *M. brunneum* conidiospores (10^8 conidiospores ml^{-1}), which was optimal for measuring worker fungal inhibition. The antifungal assay was otherwise run exactly the same as for the queens.

Data analysis
All statistical data analysis was carried out using R version 3.3.2 [230]. The raw levels of transmittance measured at time point 0 were subtracted from the levels recorded after one day of incubation with queen/worker samples, in order to remove the effect of any minor differences in initial transmittance between the samples. For workers, the transmittance values were divided by the mean worker weight to control for differences in worker size. One-way ANOVAs were used to compare the weight and total number of brood produced by queens, as well as the antimicrobial activity of queens and workers, having ensured that the data satisfied the assumptions of the tests (i.e. Cook's distance, dfbetas, dffits, heteroscedasticity and the distribution of residuals). For the antimicrobial activity of the queens, we included the individual queen weights as an additional covariate. Tukey's HSD tests were used to carry out post-hoc comparisons and the subsequent *P* values were corrected using the Benjamini-Hochberg procedure [232]. Linear regressions were used to analyse the relationship between (i) the number of workers produced by a queen and worker antifungal activity, (ii) queen antifungal activity and the total number of brood produced and (iii) queen and worker antifungal activity. For each regression, we ensured that the data met all assumptions (i.e. Cook's distance, dfbetas, dffits, heteroscedasticity and the distribution of residuals). If data were not normal or heteroscedasticity was detected (as was the case for a few regressions), the data was log transformed. All graphs were made using the 'ggplot2' R package [241].

6.3 Results

6.3.1 Brood production and queen and worker weight

The weight of the queens changed throughout the duration of the experiment (Fig. 1a; one-way ANOVA, $F_{6,77} = 10.66$, $P < 0.001$; results of post hoc testing in Table 1). Queens were heaviest after collection, but they lost approx. one third of their weight by the time they had raised pupae. Once the first workers emerged and began foraging, the queens rapidly gained weight. Interestingly, there was no loss in weight during hibernation, but a significant decrease (again, approx. one third) one year after colony founding. Queens again increased in weight by the end of the experiment. As expected, the total number of brood present in the colonies increased as they aged (Fig. 1b; one-way ANOVA, $F_{5,66} = 39.45$, $P < 0.001$; results of post hoc testing in Table 2), but there was a decrease during hibernation, where dead brood

– in particular pupae – were discarded from the colonies. The weight of the first workers and those present during hibernation were similar, however, worker weight was ~ 2.4 times higher in workers produced in the two later time points (supplemental figure 1).

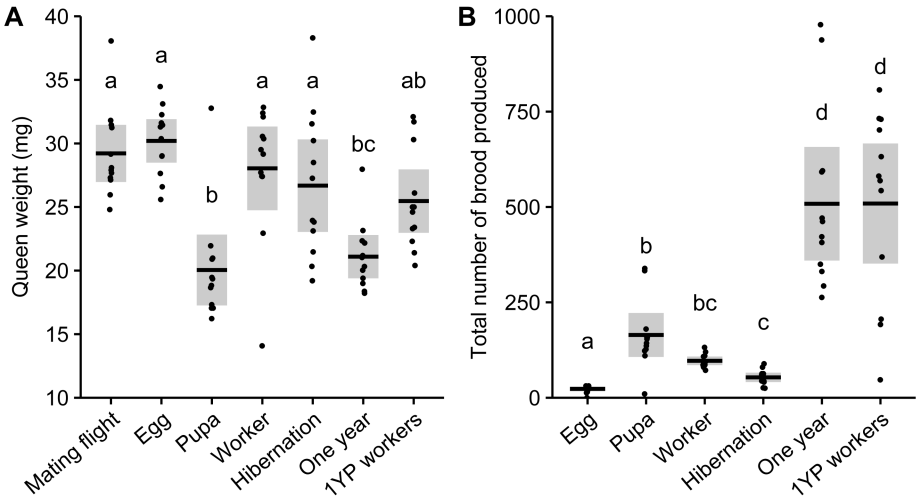

Figure 1. Queen weight and brood production. (a) Queen weight did not show a clear increase or decrease with colony growth. As expected, queens were at their lightest towards to the end of the solitary phase ('pupa' in graph). However, once the workers started foraging the queens' quickly regained weight, which continued to fluctuate over time. (b) The total number of brood (eggs, larvae, pupae and workers) produced by queens remained relatively small during the early stages of colony foundation, but increased dramatically in colonies aged ≥ than 1 year. All data points displayed; lines ± shaded boxes show mean ± 95% confidence intervals (CI); letters denote significant Tukey post hoc results at $\alpha = 0.05$. In x axis, YP = 'year post'.

6.3.2 Longitudinal analysis of queen and worker antifungal activity

Our experiment revealed that the antifungal activity of queens varied as the colony developed (Fig. 2a; one-way ANOVA, $F_{6,76} = 4.4$, $P < 0.001$; results of post hoc testing in Table 3), but was unaffected by the weight of the queen (one-way ANOVA, $F_{1,76} = 0.6$, $P = 0.44$). We found that antifungal activity increased by the time the queens laid their first eggs (approx. 1 day after the mating flight) and stayed at the same level until the first workers were produced. By this time point, the queens' antifungal activity had dropped to a level equal to queens collected after the mating flight. However, it then increased again during hibernation, before falling again in one year old queens, and, finally, increasing one year after the first workers were produced.

In contrast to the queens, the antifungal activity of the workers increased as the colony aged (Fig. 2b; one-way ANOVA, $F_{3,42} = 50.4$, $P < 0.001$; results of post hoc testing in Table 4). There was no significant difference in fungal inhibition between the first workers produced by queens and those that endured hibernation. However, the worker fungal inhibition was increased one year after colony foundation and one year after the colonies' first workers, between which there was no difference. We also tested whether there was a relationship between the number of workers produced by the queens and worker antifungal activity, but found no evidence of an effect between these two variables at any of the time points (data not displayed; linear regressions, all $P > 0.05$).

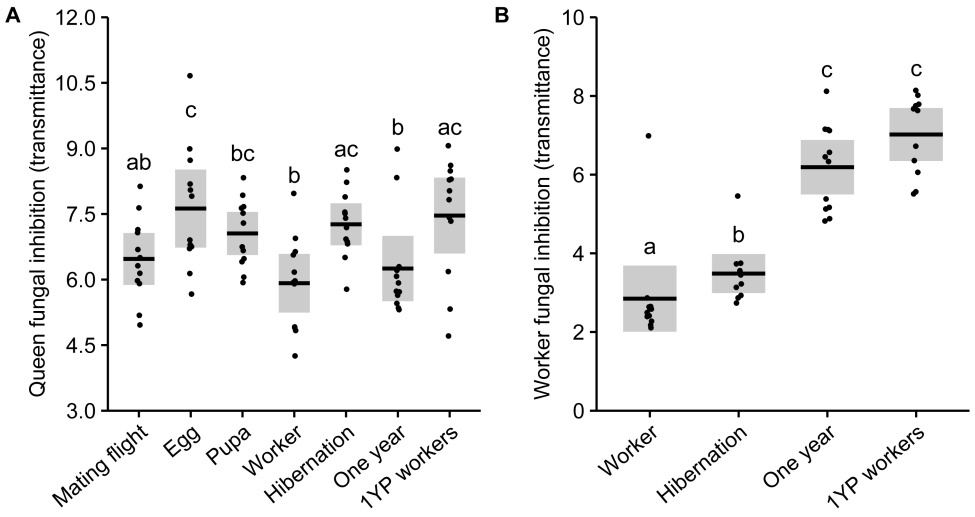

Figure 2. Immunocompetence of queen and workers with colony development. (a) The antifungal activity (fungal inhibition) of queens increased shortly after colony foundation and remained high until the first workers were produced, but then increased or decreased several times (b) Worker antifungal activity increased with colony age. All data points displayed; lines ± shaded boxes show mean ± 95% confidence intervals (CI); letters denote significant Tukey post hoc results at $\alpha = 0.05$. In x axis, YP = 'year post'.

6.3.3 Queen antifungal activity and brood production

There was no relationship between the production of brood and queen antifungal activity during the egg, worker and hibernation stages, or one year after colony foundation (Fig 3; linear regressions, all $R^2 < 0$, $P > 0.05$). However, the number of brood produced by queens before workers emerged (pupal stage) exhibited a significant, negative relationship with queen antifungal activity, with queens exhibiting greater inhibition of the fungus when producing fewer brood ($R^2 = 0.41$, $t = 2.64$, $P = 0.025$). Conversely, there was a significant, positive relationship between brood production and antifungal activity in queens one year after raising their first workers ($R^2 = 0.69$, $t = 4.46$, $P = 0.0016$). Here, the queens that raised the most brood displayed greater fungal inhibition.

6.3.4 Relationship between queen and worker antifungal activity

We could not detect any significant relationship between the antifungal activity of queens and workers at any of the measured time points (data not displayed; linear regressions, all $P > 0.05$).

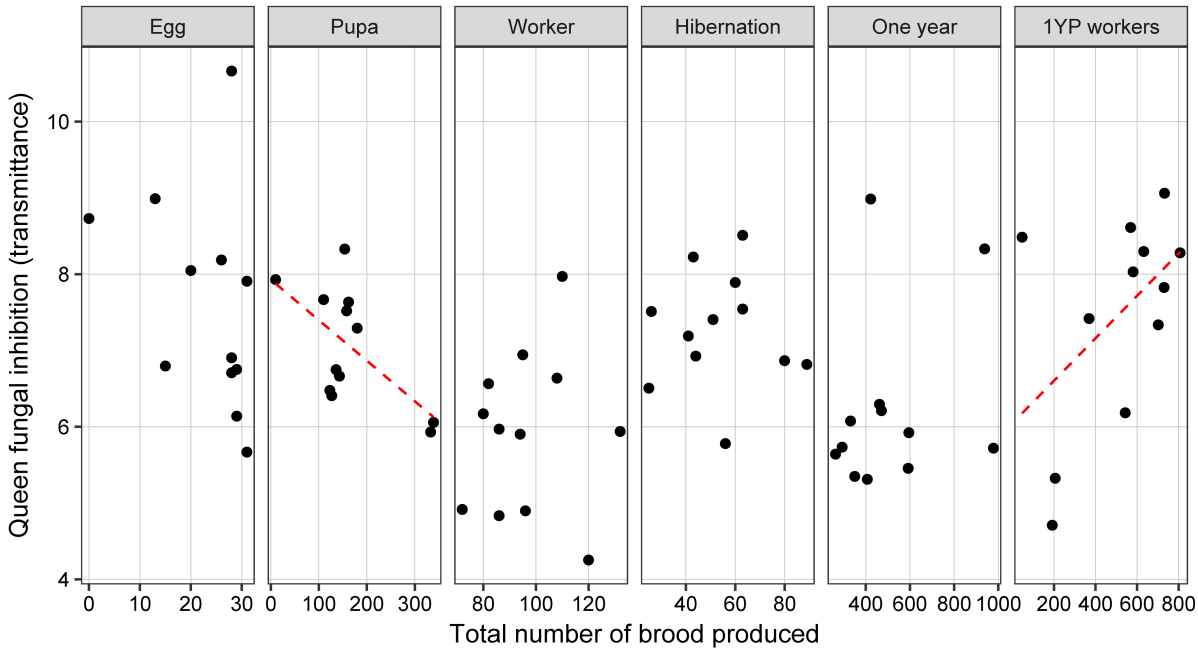

Figure 3. Queen immunocompetence and brood production. We found a significant, negative relationship between the antifungal activity of queens (fungal inhibition) and brood production at the end of the solitary queen phase of colony foundation ('pupae stage'). Conversely, antifungal activity and brood production were positively correlated in the oldest colonies measured ('1 year post workers'). Red lines depict significant fitted linear regressions. In x axis, YP = 'year post'.

6.4 Discussion

Investment into immunity should be flexible and reflect the risk of infection experienced by an organism. In ants, colonies are founded by queens that go through a stressful, solitary phase and must mate, dig a nest and raise their first workers alone [70]. Once the first workers emerge, the colony will begin growing exponentially, with foragers bringing in food and nurses that can care for the brood. As the colony grows, the risk of pathogen outbreaks will increase. However, at the same time, social immunity is emerging and may offset the costs of an increased risk of disease [17]. Hence, the immunocompetence of queens and workers is likely to change over time. Here, in our experiment on incipient ant colonies, we found that the immunocompetence of ant queens varies over time and is affected by both brood production and colony size. In addition, the immunocompetence of workers increased as colonies grow larger.

Our experiment revealed that the expression of constitutive antifungal defence is a plastic response in ant queens. In accordance with previous studies, we found that the immunocompetence of queens increases following colony foundation [164,273]. Interestingly, whereas earlier work measured immunocompetence (e.g. encapsulation response, phenoloxidase levels and antibacterial assays) for a short period after colony foundation (maximum of 9 d) – inferring that the increase was triggered by mating or infection [164,273] – our data show that, once raised, immune activity stays high during the solitary phase, and does not decrease until the first workers emerge. As previously suggested, it may be that non-lethal infections acquired during mating or searching for a colony trigger the increase in immune response [164,273]. For example, in workers of the ant *L. neglectus*, non-lethal fungal infections triggered increased expression of immune genes involved in antifungal immunity, and hence they exhibited stronger functional antifungal activity than

non-infected insects [53]. *L. niger* queens appear able to suppress infections for long periods of time [74], so the persistently high level of immune activity that we observed may have been caused by pathogen exposure whilst queens were searching for a nest, prior to collection. Indeed, 1% of the queens that we collected died from infections of entomopathogenic fungi (*Metarhizium brunneum* and *Beauveria* spp.) shortly after being returned to the laboratory, demonstrating that ants can encounter and contract lethal infections within very short timescales of leaving the protection of the nest (all queens were collected within a few hours after mating flight), or even within the parental colony. Alternatively, queens may raise their constitutive immune activity prophylactically during this period. Since queens are severely resource-limited this will be costly [164,273], and we found that the more a queen invested into immunity the fewer brood she raised before the first workers emerged. However, because queens must survive the colony founding stage if they are to have any chance of gaining fitness in the future [70], investment into immunity during the solitary phase may be adaptive, and perhaps as important as raising a sufficient initial number of workers, to compete with other incipient nests [66,76].

Following the emergence of the first workers, the antifungal activity of queens decreased and increased several times as colonies developed. On the one hand, this is surprising given that the queens are now receiving food and so should be able to invest more into immunity. On the other, the queens may have also exhausted their fat and muscle reserves by this time and so were not able to invest into immunity any longer [77,80]. At the end of hibernation the queens exhibited an increase in immune activity. Because temperature can affect metabolism and the effectiveness of immune responses [281], it is not unexpected to observe differences in immunocompetence during hibernation; however, warmer temperatures typically improve the effectiveness of immunity [291], rather than cooler ones, as we observe here. There was an additional decrease in immunity one year after colony foundation that might be explained by queens investing more into reproduction, however, by the final time point, immunity was once again increased. Further studies are needed to investigate the exact cause of these fluctuations, but investment into immunity is likely to be dependent on the condition of queen and her reproductive state [281]. For example, social insect queens exhibit bouts of physogastrism throughout spring and summer, where the ovaries swell and egg laying intensifies [283]. Changes in reproductive activity could explain why immunity was decreased one year after colony foundation (mid summer), but increased again two months later (autumn). At this point, queens should stop laying eggs and start storing resources for hibernation. Indeed, this is the pattern we observed in the weights of the queens.

Animals can opt to produce many lower quality offspring or few higher quality ones, and the optimal solution is likely to depend on the resources available to the mother [292,293]. Here, we found no evidence of a “quantity-quality” trade-off between the number of workers a queen produced and worker antifungal activity, but, overall, worker antifungal activity increased as colonies aged – a pattern that has been observed in bumblebees and leaf cutting ants as well [279,288]. In this case, the most logical explanation for the observed increase in antifungal activity is improved nutrition and brood care, because, as the number of workers in the colony increases as it ages, there are more foragers to collect food and nurses to care for the brood. Indeed, we found that the dry weight of workers also increased as colonies aged, indicating that they were likely receiving better nutrition towards the end of the experiment than at the beginning, where nanitic workers were raised on the limited resources of the queen [283].

However, another possible explanation for the increase in worker antifungal activity is density-dependent prophylaxis (DDP), where animals are hypothesised to invest positively into constitutive immunity when the number of conspecifics is high, due to the greater risk of infection in denser social groups [294]. Empirical evidence for DDP shows that this expectation holds true [280,295,296], and, similar to our study, bumblebees and leaf-cutting ants have been shown to exhibit a similar pattern, with the levels of constitutive immune defences increasing in workers with colony age, and thus colony size [279,288]. Indeed, another study that supports this observation found that DDP in bumblebee workers is an extremely plastic response, which can operate within very short time frames [278]. DDP could also explain why the eldest queens in our study exhibit a positive relationship between the total colony size and antifungal activity, despite the presence of social immunity defences performed by workers that should reduce their exposure to pathogens and, thus, risk of infection [17]. However, since social insect colonies go through dramatic changes in colony size throughout the seasons [282], being able to plastically adjust immunocompetence may be beneficial and adaptive to prevent disease outbreaks [278].

To conclude, the immune activity of ant queens fluctuates throughout the early stages of their lives and likely reflects both the environmental and physiological changes they experience. Our study and others have shown that the immune activity of workers increases as a function of colony age and, hence, colony size [278,279,288]. Whereas previous studies have found evidence of DDP in workers, our results show that queens may also up-regulate their immune activity in a density-dependent manner. Unlike the sterile workers, however, she is likely to continue to experience fluctuations in immune activity, given that she goes through cycles of egg-laying each year, which may continue to cause reproduction-immunity trade-offs [283]. Despite the diverse and effective social immunity defences present in ant colonies [17,43,284], our results and others suggest that the innate immune still plays an important role in colony resistance to disease [278,288]. Yet, whilst our study covered a significant portion of early stages of colony development compared to previous work, sexually mature colonies with hundreds of thousands (if not millions) of workers are still likely to experience different disease pressures, and further work is needed to gain a fuller picture of immunity and social immunity in social insects [284].

6.5 Acknowledgments

We thank Anna V. Grasse and Meghan L. Vyleta for establishing the fungal characterisation methodology, Miriam Stock and Matthias Konrad for establishing the antifungal assay methodology and the whole *Social Immunity* group at IST Austria for helpful discussion of the project and assistance feeding colonies.

Tables

Table 1. Tukey post hoc comparisons of queen weight between different stages of colony development. Following a one-way ANOVA showing a significant effect of colony stage on queen weight, we performed Tukey post hoc comparisons to determine which stages differ. All *P* values have been corrected for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.05$; ns = non significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Post hoc comparison	Adjusted *P* value	Significance level
Egg vs. mating flight	0.99	ns
Pupa vs. mating flight	< 0.001	***
Worker vs. mating flight	0.99	ns
Hibernation vs. mating flight	0.75	ns
One year vs. mating flight	< 0.001	***
1YP workers vs. mating flight	0.30	ns
Pupa vs. egg	< 0.001	***
Worker vs. egg	0.86	ns
Hibernation vs. egg	0.38	ns
One year vs. egg	< 0.001	***
1YP workers vs. egg	0.092	ns
Worker vs. pupa	< 0.001	***
Hibernation vs. pupa	0.004	**
One year vs. pupa	0.99	ns
1YP workers vs. pupa	0.032	*
Hibernation vs. worker	0.98	ns
One year vs. worker	0.002	**
1YP workers vs. worker	0.74	ns
One year vs. hibernation	0.024	*
1YP workers vs. hibernation	0.99	ns
1YP workers vs. one year	0.15	ns

Table 2. Tukey post hoc comparisons of total amount of brood produced between different stages of colony development. Following a one-way ANOVA showing a significant effect of colony stage on the total amount of brood produced, we performed Tukey post hoc comparisons to determine which stages differ. All *P* values have been corrected for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.05$; ns = non significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Post hoc comparison	Adjusted P value	Significance level
Pupa vs. egg	< 0.001	***
Worker vs. egg	< 0.001	***
Hibernation vs. egg	0.01	**
One year vs. egg	< 0.001	***
1YP workers vs. egg	< 0.001	***
Worker vs. pupa	0.85	ns
Hibernation vs. pupa	0.01093	*
One year vs. pupa	< 0.001	***
1YP workers vs. pupa	0.00153	**
Hibernation vs. worker	0.21	ns
One year vs. worker	< 0.001	***
1YP workers vs. worker	< 0.001	***
One year vs. hibernation	< 0.001	***
1YP workers vs. hibernation	< 0.001	***
1YP workers vs. one year	0.99	ns

Table 3. Tukey post hoc comparisons of the antifungal activity between different stages of colony development. Following a one-way ANOVA showing a significant effect of colony stage on the antifungal activity of queens, Tukey post hoc comparisons were performed to determine which stages were driving this significant effect. All *P* values have been corrected for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.05$; ns = non significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Post hoc comparison	Adjusted P value	Significance level
Egg vs. mating flight	0.047	*
Pupa vs. mating flight	0.55	ns
Worker vs. mating flight	0.24	ns
Hibernation vs. mating flight	0.19	ns
One year vs. mating flight	0.53	ns
1YP workers vs. mating flight	0.16	ns
Pupa vs. egg	0.32	ns
Worker vs. egg	0.004	**
Hibernation vs. egg	0.55	ns
One year vs. egg	0.021	*
1YP workers vs. egg	0.62	ns
Worker vs. pupa	0.10	ns
Hibernation vs. pupa	0.55	ns
One year vs. pupa	0.14	ns
1YP workers vs. pupa	0.53	ns
Hibernation vs. worker	0.02	*
One year vs. worker	0.69	ns
1YP workers vs. worker	0.01	*
One year vs. hibernation	0.046	*
1YP workers vs. hibernation	0.88	ns
1YP workers vs. one year	0.03	*

Table 4. Tukey post hoc comparisons of the antifungal activity of workers between different stages of colony development. A one-way ANOVA found a significant effect of colony stage on the antifungal activity of workers. We performed Tukey post hoc comparisons to determine which stages differed. All *P* values have been corrected for multiple testing using the Benjamini-Hochberg procedure ($\alpha = 0.05$; ns = non significant, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Post hoc comparison	Adjust P value	Significance level
Hibernation vs. worker	0.013	ns
One year vs. worker	< 0.001	***
1YP workers vs. worker	< 0.001	***
One year vs. hibernation	< 0.001	*
1YP workers vs. hibernation	< 0.001	***
1YP workers vs. one year	0.16	*

7. Towards a unified framework for studying disease defences

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Author contributions
Conceived topic: DF
Literature review: CDP, DF
Wrote topic: CDP, DF

Sections in detail
The problem: CDP (50 %), DF
Conceptual advance: CDP (50 %), DF
Transgenerational immune priming: DF
Costs of immune defence: DF
Fitness effects of behavioural disease defences: CDP (100 %)
Social networks: CDP (100 %)

7.1 The problem

Research on the vertebrate adaptive immune system has led to major advances in our understanding of host disease defence [7]. However, despite the potency of immunological memory, it is often innate immunity that makes the difference between survival and death [297]. The discovery that invertebrates, including social insects, can prime their immune responses despite lacking adaptive immune machinery, and are able to transfer immune protection across generations, is paradigm-shifting [167,298,299]. Further, many animals, including humans, live socially, and there is an emerging view that social interactions can both negatively and positively affect disease dynamics [116,300]. For example, although living in groups can be costly because susceptible and infectious animals will more frequently interact than in solitary species, cooperation and the evolution of collective behavioural defences can enhance disease resistance and tolerance in social animals [5]. In general, we lack a solid understanding of the interplay between physiological immunity, behavioural responses and social interactions, yet this is essential to the study of disease defence, host-pathogen coevolution and epidemiology [7].

7.2 Conceptual advance

Social insects offer an integrated approach for the study of disease defence. Unlike other animal societies, social insect colonies can be easily observed and manipulated in the laboratory and field, with high levels of replication. The natural variation and plasticity present in their colonies makes them ideal candidates for ecoimmunological work and powerful tools to study the evolution of immune defences from the gene to the society [97]. With the diversity of social complexity in social insects, we can study disease dynamics and epidemiology in societies of different sizes and social structures. Studying disease dynamics in social insects has already led to new concepts: “social immunity” is the additional layer of defence arising from collectively performed disease defences that reduce the disease susceptibility of superorganismal social insect societies, rather than its individuals [17]. It is thereby analogous to the physiological immune system of multicellular organisms, and could provide insights into the evolution of immune defences across these domains [51].

7.3 Challenges and Solutions

We identify four critical issues that are of general importance to the study of disease defence in animals.

7.3.1 Transgenerational immune priming

The first concerns the molecular mechanisms underpinning transgenerational immune priming without antibodies. The fact that mothers can enhance the resistance of their offspring against diseases without antibodies has been a puzzle and we are only beginning to understand the mechanisms behind it. For example, a mechanism for maternal immune priming in insects was recently discovered in the honeybee: egg yolk protein vitellogenin transfers bacterial fragments from food to developing eggs [168,298]. However, we do not yet know what other mechanisms are at play. Do mothers also pass on other molecules (e.g. mRNA or specific proteins), which shape the phenotype of the developing embryo? It has also been argued that the evolution of immune priming in insects is dependent on the longevity of the species and mechanism of dispersal [301], but strong evidence is lacking. Social insects would be ideal candidates to test these hypotheses as they exhibit large variation in longevity and dispersal strategies.

7.3.2 Costs of immune defence

Secondly, a better understanding of the costs of immune defence is required. There is suggestion that resistance is traded off against other fitness traits and depends on life history [7]. Immune priming gives undeniable fitness benefits in the case of re-infection, but the costs are largely unknown [302]. In social insects with a strict colony structure and task division, we predict that the costs and need for immune defence would differ between colony members. With well-established model systems in social insects and a growing availability of genomic information, we are able to pinpoint the costs immune defences inflict on fitness related traits, the interaction between physiological and behavioural immunity, as well as what kind of costs are linked to behavioural defences.

7.3.3 Adaptive importance of behavioural disease defences

Thirdly, the adaptive significance and fitness effects of behavioural disease defences are untested or unknown in most cases. In order to study the impacts of behavioural defences on survival and fitness, longer term studies are required and can be achieved using social insects with short generation times (e.g. [105]). Experimental manipulation of behaviour can be challenging, but is possible in the social insects. As an example, nest entrances can be closed to prevent the removal of corpses, a key social immunity behaviour [138]. Moreover, with the increasing number of sequenced genomes and RNAi-mediated knockdown techniques, it is becoming more accessible to directly target genes that affect behavioural phenotypes [303].

7.3.4 Social networks

Finally, studies of complete social groups are needed to understand the role of social networks on disease transmission and susceptibility [122]. Social insects are ideal candidates for studies of epidemiological networks, given whole colonies can be observed and manipulated, and they exhibit modulatory in the form of morphological castes and performance of tasks [44,120,304]. With the development of tracking techniques and advances in network analysis, disease outbreaks can be studied across space and in real-time, providing fine-scaled monitoring of behaviours and changes in social interactions [122].

8. Conclusion

The overall aim of this thesis was to understand how social immunity defences mitigate the impact of successful infections when initial barriers to disease fail to prevent infection. Additionally, I set out gain a greater insight into disease defence and immunity during the early stages of colony development.

In the second chapter, we aimed to summarise how the field of social immunity has advanced since the coining of the term a decade ago [17]. During this time period, the field has expanded and the concept has been extended to non-eusocial insect systems, e.g. transient parent-offspring associations and other animal aggregations [5,114,115]. Although both the latter are interesting areas of study, we feel that broadening the term to include systems in which the level of selection is still preferentially at the individual, rather than the social group, dilutes its usefulness as a framework to understand how collective disease defences evolve, which benefit the social group over the individuals that make it up [17,42,43]. This is because as only in these situations can cooperation be great enough, and conflict low enough (caused by the high relatedness among individuals), for altruistic social immunity to be selected for, as e.g. self-destructive behaviours, via indirect fitness benefits [113]. We believe that this aspect is crucial for understanding how social immunity functions and evolves and makes it comparable to organismal immunity, where selection acts also at the level of the reproductive unit. Parallels have been drawn between the immune system of multicellular organisms and social immunity in superorganismal societies for some time [17,51]. Whether these analogous systems are an example of convergent evolution at two levels of biological complexity remains an open and hard-to-address question [32,50]. But, by clearly defining social immunity as 'the immune system of the colony', we may be able to use models, comparative analyses and experimental manipulation, to understand under what conditions social immunity evolves.

Echoing the work of previous authors [43], we also propose that the avoidance, resistance and tolerance framework can be applied to social immunity. Most studies on social immunity have focussed on disease resistance (e.g. [103,140,146,191,201,202,228,305]), and a few have demonstrated avoidance in social insects (e.g. [107,125]). However, tolerance has been almost completely neglected [306]. Following the study of tolerance in whole organisms [30,155], we propose that by viewing social insect colonies as single organisms, the same predictions and tests can be made about tolerance in social immunity. For example, different worker groups within the colony may be more or less tolerant to infections than others. Moreover, colonies may opt for tolerance as a strategy over resistance.

Lastly, we focussed on how social immunity may affect the evolutionary outcomes of host-pathogen interactions. In particular, we predict that, for generalist pathogens, social insects are suboptimal hosts and most likely represent "dead ends" for the pathogen, as social immunity defences can efficiently prevent successful infections from transmitting to new hosts [56,109,111]. However, the abundance of these pathogens in the environment may apply a persistent selection pressure on the social hosts to evolve capable social immunity defences – hence, we should see the accumulation of one-sided adaptations in the host, against a variety of commonly encountered pathogens [307].

In the third chapter, I characterised a previously unknown, multicomponent behaviour termed "destructive disinfection", which we observed in the invasive garden ant *Lasius neglectus*. Through a series of linked experiments, I show that this behaviour is distinct from the

sanitary care performed by ants towards pathogen-exposed, but not yet infected, brood [55,144,192,202]. Indeed, destructive disinfection is complementary, forming a second barrier that can prevent disease transmission when sanitary care fails to prevent a lethal infection. In the end, destructive disinfection prevents the pathogen from producing transmissible stages, essentially reducing its fitness to zero. Because the R_0 of the pathogen is diminished, such behaviour may explain why infections of generalist pathogens are rare in ant colonies, despite them being present in high abundances in the environment [46–48]. Moreover, such strong selection pressure on ant-infecting pathogens could have led to the remarkable level of host-manipulation observed in ant specialist fungi, which are able to infect ant colonies for sustained periods of time [104,222]. Hence, as suggested in chapter two, linking social immunity behaviours to host-pathogen dynamics may help to explain observed patterns of disease in the field.

Destructive disinfection is triggered by changes in the chemical profile of infected pupae. Interestingly, two of the hydrocarbons we identified on sick pupae also increased with infection in honeybees [108,128]. This suggests that these cues may have evolved into "sickness signals" in the Hymenoptera. Although beyond the scope of this thesis, synthesising these compounds and performing a comparative analysis with different species of social insect may help to establish this link, similar to studies on queen pheromones [308]. Furthermore, additional investigation into the underlying mechanisms leading to the increase of these compounds could shed light on whether the observed changes are cues or signals.

In the fourth chapter, I showed that *L. neglectus* sprays its poison frequently over large areas of the nest, which is inline with previous studies showing, indirectly, that ants use their in the nest, as objects taken from and placed into nests are coated in poison [55,136]. Prophylactic sanitation of the nest environment may be important in ants and other social insects, as they often live directly in the soil, which can contain a high diversity and load of pathogens [46–48]. Hence, regular systemic sanitation, through the production of chemicals, symbionts or environmentally acquired substances, should aid in keeping pathogen abundance low and the risk of infection minimal [137,141,309,310].

The antimicrobial activity of endogenously produced antibiotics has increased with sociality in bees, demonstrating that there is a correlation between the risk of infection and the need for effective disease defences [311]. However, the antimicrobial activity of these secretions cannot continue to increase linearly, and is likely constrained by the metabolic cost of synthesis [255], the harm they can cause the producer during storage [157], and, potentially, the damage they may cause to other colony members. Having observed in chapter two that the cocoon prevents the ants' poison reaching the pupae inside, I hypothesised that the cocoon may protect developing pupae from prophylactic nest sanitation. Through several experiments, I provide evidence that the cocoon does indeed protect *L. neglectus* pupae from ant poison spraying.

Spinning a cocoon appears to be costly and slows the development of the pupae [166]. The data I present is therefore interesting as the results hint that poison spraying could be selecting for the maintenance of the cocoon in *L. neglectus*, especially as the cocoon has been lost in most other species, which do not produce acidic poison. Yet, within the poison spraying Formicines, some species also lack cocoons. Hence, comparative work is needed to establish cause and effect. However, at least in *L. neglectus*, spinning a cocoon may relax constraints on the toxicity of the ants poison, by ensuring it cannot cause harm to vulnerable colony members.

In the fifth chapter, I describe the undertaking behaviours of ant queens and discuss how these behaviours may influence colony-founding success, using queens of ant *L. niger*. I show that queens were equally like to found colonies with pathogen-exposed conspecifics as they were with sham-treated queens, and that their own health state does not affect their decision. As in termites that must choose a mate to start a colony with [264], other pressures, such as desiccation and predation, may outweigh the costs of being choosey when co-founding. Indeed, co-founding in ants may be caused by a constraint on available nest sites by overcrowding, which could reduce selection on queens to choose an appropriate co-founder.

Previous work has shown that co-founding queens do not engage in social immunity behaviours, probably because they are usually unrelated and should therefore not risk contracting infections themselves [74]. However, here I show that when a co-foundress dies, queens will perform undertaking behaviours towards the corpse. Specifically, the queens were observed biting, burying and removing the corpses. These undertaking behaviours were occurred prophylactically towards both infected and non-infected corpses, but before the pathogen became infectious. The chances of survival were increased when queens performed some of these behaviours, suggesting that they may be adaptive.

Since colony-founding queens must survive on the body reserves that they leave their parental colony with, they have to balance investment into life history traits to maximise fecundity [70]. For example, queens exposed to *Metarhizium* may survive infection but produce an initially smaller number of workers, which will place them at a disadvantage compared to other colonies [74,76]. Behavioural traits that can prevent infection may therefore be under strong selection in queens, as they are likely cheaper and less risky to perform that triggering an immune response pre or post infection. However, the innate immune response of queens is likely to still play an important role in protecting them during the solitary phase of their lifecycle, but how it importance as colonies grow and develop is not understood.

To that end, in the sixth chapter, I explored how the immunocompetence (measured as antifungal activity) of queens and workers changes as incipient ant colonies grow and develop. In addition, I measured the weight of queens and the number of brood produced to determine if immunocompetence trades-off with fecundity. I found that queen antifungal activity was a dynamic process, regularly increasing and decreasing. Inline with previous studies, queens initially increase their antifungal activity, which may be an adaptive response to the lack of workers that can protect her from disease and increased risk of infection. As expected, queens that invested more into immunity during the solitary phase produced less brood in total, a trade-off that has been observed in many animal taxa [263,312–314]. Once the first workers emerged, queen antifungal activity fluctuated several times, but, interestingly, was correlated with colony size at the final time point. In addition, worker antifungal activity increased with colony size as well. This is similar to results obtained from studies in bumblebee and leaf-cutting ant workers [278,279,288], which may be explained by density-dependent prophylaxis (a general increase in immunity with group size) [294].

In the final chapter, D. Freitak and I have highlighted the usefulness of social insects as a model organism for studying the evolution of host defences against disease. In social insects, disease defence can be studied at both the molecular, cellular, individual and society level [97], offering the unique opportunity to understand how these different levels interact and trade-off against one another. As more tools are becoming increasingly available for studying non-model organisms [44,195], the social insects will play an important role in linking several diverse fields, including immunology, behavioural ecology and epidemiology.

9. Outlook

In chapter two, providing a more focussed definition of the term social immunity may help in making social insects a model system for understanding how "immune systems" evolve as new levels of individuality emerge. Social insects are ideally suited to study this given the diversity of social complexity present across their lineages [315]. For example, some social insects, such as the termites and wasps, are comprised of both cooperative breeders and superorganisms, making them particularly suited for comparative analyses across these two types of social organisation, which may provide an insight into what factors drive the emergence of social immunity. For example, cooperatively breeding species, such as *Polistes* wasps, often vary in the relatedness of individuals within colonies [316], making them potentially interesting organisms to study whether social immunity can be conditionally expressed when colonies are comprised of mainly relatives, but not non-relatives. By taking such an approach, we may be able to understand in more detail the conditions that are necessary for social immunity to evolve. In turn, this could shed light on the processes involved in the evolution of immune systems, which, analogous to social immunity, protect multicellular individuals from disease.

In the third chapter, I have showed that destructive disinfection is an efficient behaviour that appears to be adaptive in preventing disease spread in colonies. This work extends our current understanding of how social insects cope with disease, demonstrating that, in addition to simply isolating infected individuals [143,149], ants can also actively prevent pathogens from growing and replicating. As a consequence, destructive disinfection and similar behaviours, such as cannibalism in termites [109,201], should have influenced the evolution of pathogens infecting social insects, since it will apply a strong selection pressure on pathogens to overcome this defence [284]. Hence, as a next step, it would be interesting to study how destructive disinfection actually affects pathogen evolution. Co-evolution experiments are difficult, if not impossible, to perform with social insects, but one-sided adaption of pathogens to a host could be performed [8]. Since a small percentage of pupae (~ 5%) still sporulate and become infectious despite pupae being destructively disinfected, it should therefore be possible to culture those fungi and carry out serial infection passages of new pupae to determine if, and how, the pathogens might adapt to overcome this behaviour. For example, one possible way of overcoming destructive disinfection might be to evolve a higher tolerance towards the poison of the ants. Such a study may elucidate the mechanisms that allow specialist fungi, which cause chronic colony infections, to overcome social immunity defences [104,223], or demonstrate why generalist pathogens fail to cause significant disease outbreaks in ant colonies, despite their ubiquity in the environment [284]. An alternative approach to a one-sided adaptation experiment would be to grow fungi in media with artificial ant poison, for several generations, which could address similar questions.

In general, destructive disinfection could become a useful 'tool' as a standardised measure to study various aspects of social immunity in ants. Currently, we know it is performed by several species with cocoons and is likely present in many more, including those without cocoons. Given the outcome of destructive disinfection is binary – it is either performed or it is not – it is a simpler behaviour to quantity than grooming, which has previously been used to carry out comparisons of disease defence across ant species, but is extremely variable, both between colonies and ants of the same colony [107,149]. This variation would be interesting to study, but with grooming it is hard to determine how much variation is caused by differences in the amount of conidiospores ants' receive, which is likely to be influenced by

factors such as differences in body shape and size, cuticle thickness, levels of activity and interaction rates, and others. On the other hand, pupae are more uniform in shape, which should reduce variation in the pathogen exposure step. Furthermore, because destructive disinfection is a binary measurement, is should be less influenced by individual-level variation between ants, which could possibly skew quantitative measures such as grooming duration (e.g. because some ants simply groom more than others). In addition to the presence/absence of destructive disinfection, other measures can also be quantified, such as the time until unpacking occurs, which will be affected by both the strength of the signal expressed by the pupae and the ability of the ants to detect it. Additionally, an important measure is how successful destructive disinfection is, which is again a simple binary read out – do the pupae sporulate or not?

Examples of experiments where destructive disinfection could be a useful measure include assessing how much social immunity varies between colonies, using destructive disinfection as a proxy. Understanding how much colonies vary in social immunity adaptations is an important question that is currently severely understudied – in most cases, colony is included as a random effect, so that generalisations can be made about the observations in question [150,187,192,317]. However, understanding the cause of both intra- and inter-colony variation could prove an exciting avenue of research – is it caused by differences in the types of workers used, e.g. foragers versus nurses, the ability of ants to respond to disease cues, or, importantly, due to life history trade-offs with other traits? The latter is most tantalising, given we currently lack information about the costs of performing social immunity [315]. Yet, social animals are expected to invest more into disease defences than solitary individuals and performing social immunity behaviours will distract insects from other tasks that affect fitness, such as foraging or feeding the brood. In sum, destructive disinfection offers as a simple, robust read out that could be used comparatively to address important gaps in our current understanding of social immunity.

In the fourth chapter, I show that cocoons protect developing pupae from poison use in ant nests. Until this study, only indirect evidence of poison use throughout the nest was known [136] and it has even been previously speculated that applying poison orally, as invasive garden ants do during sanitary care, should be favoured to prevent poison being wasted [55]. However, given we see large quantities of poison being sprayed regularly in the nest, perhaps the poison is not such a limited resource after all, especially in large colonies with many workers. Instead, applying poison orally may serve a different purpose: reducing the damage it can cause to brood and workers, given its cytotoxic qualities [157]. In support of this, in chapter three, I show that ants do spray large quantities of poison onto pupae, but only when they are already doomed to die.

One potential limitation of the work in chapter four is that poison spraying was only studied in ants moving into new nests. Whilst this does not affect our conclusions about the protective effect of the cocoon (given pupae are frequently moved around chambers), it is possible that poison spraying may be less in established chambers. Hence, it would be interesting to study the temporal-spatial use of poison in multi-chambered ant colonies, over time, where new chambers are added periodically to simulate nest expansion. Using such an experimental set up, we may find that, indeed, only new chambers are treated with large amounts of poison. A second drawback of this study is that we focussed only on the effect of the cocoon on pupal survival. Hence, we are unable to comment on the effect of poison on the eggs and larvae, which are left ‘bare’ in colonies, unlike the pupae, and so should regularly come into contact with the poison. Differences in the physical structure of the eggs

and larvae could protect them from poison spraying, such as the presence of a chorion around the eggs and the fact that larvae regularly undergo ecdysis, which may allow them to shed any damaged cuticle. Additionally, it is possible that the ants use their poison selectively, avoiding areas of the nest where eggs and larvae are present, given that they are typically kept separate from the pupae [251]. Again, studying the spatial dynamics of poison spraying across larger nests would be able to address this latter hypothesis.

Despite these limitations, this study is one of the few to address the impact of colony-level defences, such as global nest disinfection, on other individuals in the colony, and how this may then interact with other life history traits – in this case, the potential maintenance of cocoon spinning in the larvae. Such evolutionary relationships are likely to be common: a recent study found that the presence of a functional sting affects the presence or absence of other defensive traits, such as body spines, colony size etc. [318]. So far, the trade-off of social immunity behaviours with other colony traits has not been assessed, but poison spraying, similar to the presence of a sting, should be energetically costly to maintain and the presence of poison spraying within the nest should itself have evolutionary consequences. It is conceivable that poison spraying species might have thicker cuticles, nest structures with improved ventilation or, as our data suggest, the presence of cocooned pupae.

An interesting, but as yet, unstudied aspect of poison use in the colony is how social insects overcome the issue of antimicrobial resistance. Given that the poison is used both to actively kill pathogens during infections (chapter three) and as a broadband surface disinfectant in the nest (chapter four), it is likely to shape the microbial communities both in the nest and the surrounding environment (for example, the soil surrounding wood ants nests has a lower pH, potentially caused by poison spraying [319]). Can pathogens evolve in response to the use of poison and what stops pathogens from evolving resistance to it? By studying this aspect of social immunity, potentially using the abovementioned one-sided adaptation approach, it may be possible to tease apart these evolutionary interactions and potentially shed light on how animals overcome the issue of antibiotic resistance in the wild, a problem currently facing human societies [320].

In the fifth chapter, I characterised the undertaking behaviours of co-founding queens. Similar to workers, colony-founding queens dismantle the corpses of their co-founders and bury them. When possible, they will also remove them from the nest. The first two behaviours reduced the likelihood of the queens contracting an infection and dying themselves. However, surprisingly, we found that removal from the nest did not affect survival. This is likely due to the small sample size for queens that did not perform the behaviour, but the role of necrophoresis requires further investigation. For example, the experimental set up of our study made it hard for us to determine whether queens actually perform necrophoresis in the wild. An alternative way to assess this in the lab is to use a more natural set up, where queens have to dig into a substrate that is sandwiched between two plates of glass [321]. Using this set up, we would be able to see whether queens do indeed entirely remove corpses from their chambers to the surface of the nest, or whether they dig additional chambers to place them in. Of course, when the option to move them is not as easy to perform as in our experimental set up, we might observe queens biting and bury the corpses instead, given digging is energetically costly and reduces colony founding success [267]. With such a set up, it might also be possible to determine what influences the kinds of behaviours that we observe. Do queens opt for necrophoresis when they have built shallow nests verses deep ones? Does the density of the substrate influence the effectiveness of burial behaviour? Our study has characterised the behaviours queens can express, but it could be

extended further to gather more information on when and why certain behaviours are expressed. Overall, we also still lack an understanding of how colony founding is influenced by pathogens. For example, species or populations living in areas with greater pathogen pressure might be under selection to co-found more or less often, given the potential positive [74,80] and negative effects (as shown in chapter five) of having multiple foundresses in the nest when queen's are faced with disease. Hence, more studies that examine the role of pathogen during colony foundation may help shed light on the selective pressures that have led to the presence or absence of co-founding, which is seen within the ants.

In the final experimental chapter, I examined how immunocompetence develops and changes as colonies mature from single queen, founding colonies to colonies in the ergonomic growth stage with several hundred workers. This study is still research in progress, but initial results show interesting changes in the immunocompetence of queens over time, which does not progress linearly as a function of time. However, workers do increase in immunocompetence over time. The next steps for this study are to examine bacterial inhibition, in addition to the current fungal inhibition, to determine whether this follows a similar pattern or whether there are trade-offs between the two, which might change as colonies grow. For example, during the early stages of colony growth where the queen is alone with her brood and does not feed, the major source of mortality is likely to be primarily soil-borne fungi [79,81]. However, as the workers emerge and the colony starts growing – and with it food sharing and waste production – the risk of oral bacterial infections could begin to increase. Hence, we might expect to see changes in how both the queen and workers invest into antifungal and antibacterial immune defences. Addressing how immunocompetence changes over the development of a colony is an interesting question, given it should help to shed light on the relative importance of the individual ants' immune systems and social immunity, which could trade-off with one another given they are both going to be costly to maintain. Though this study only focuses on one half of this trade-off, the immune system, it will hopefully lay the groundwork for future studies that assess how social immunity develops as colonies grow, which will likely require whole-colony tracking and a diverse set of behavioural experiments to assess [284].

10. References

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