Social organisation and pathogen transmission in African ants: at what point do social immunity benefits diminish?

By

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Dissertation presented for the degree of Doctor of Philosophy in the Faculty of Science at Stellenbosch University.

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March 2020

Declaration

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Abstract

Eusocial insects, and ants in particular, encounter a range of pathogens, often generalist entomopathogenic fungi that profit from their hosts' dense living conditions and high relatedness. Ants exploit a range of individual behaviours that ameliorate pathogen impacts on the colony, collectively termed "social immunity". Species with different life histories and ecologies combat fungal infections using different approaches. This study assessed a range of social immunity mechanisms employed by three South African ant species, *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre*. Self-grooming, allogrooming, trophallaxis, and organisational immunity, as well as the effect of group size, were assessed through a range of methods including group level observations, colony level observations, spatial use assessments, interaction networks, and mathematical models. In assessments of group level effects, I showed that as group size increases ants increased their investment in grooming to remove conidia. Further, I showed that all three species responded to pathogen exposure by increasing interaction rates and mitigated exposure to the generalist entomopathogenic fungus, *Metarhizium anisopliae.* Mortality did not differ 21 days post-exposure compared to control treated ants, with access to nestmates and social immune interactions. Each species drastically lowered the number of conidia on their cuticle if allowed to groom; however, *C. fulvopilosus* displayed ineffective allogrooming. Commonly, ants increased the frequency and decreased the duration of allogrooming in response to exposure. Species displayed differential response in these behaviours with *A. custodiens* grooming most frequently and *C. fulvopilosus* allogrooming the least. The duration and frequency of trophallaxis was maintained in response to exposure, with *A. custodiens* engaging in the most trophallaxis, in terms of both frequency and duration. Assessing organisational immunity in the form of spatial use patterns, I showed that all three species displayed clustering within nests, likely limiting pathogen transmission. Only *A. custodiens*, however, showed spatial separation between foragers and the queen and further increased clustering in response to exposure, limiting pathogen spread. I generated interaction networks for *C. fulvopilosus* obtaining data from behavioural recordings of experimental colonies and calculated network metrics before and after pathogen exposure. *Camponotus. fulvopilosus* decreased network connectivity in response to pathogen exposure which limits pathways for pathogen spread. Finally, I generated matrix projection models based on the data to assess how each of the three species managed exposure to fungi, by tracking spores as they are managed by self-grooming and allogrooming.

All three species were able to mitigate pathogen exposure, removing all conidia before they could lead to infection. *Anoplolepis custodiens* relied primarily on allogrooming whilst *C. fulvopilosus* relied only on self-grooming to remove conidia. *Tetramorium sericeiventre* relied primarily on self-grooming but also benefitted from allogrooming. Overall, I show that three African ant species mitigate exposure to a generalist entomopathogenic fungus through a different combination of behavioural social immunity mechanisms, highlighting the importance of assessing several pathogen control mechanisms across multiple species. This represents the first assessment of social immunity in South Africa, showing that three species use either individual or collective behaviours to mitigate fungal exposure.

Acknowledgements

This dissertation would not have been possible without the funding provided by the National research fund (NRF) and the South African Centre for Epidemiological Modelling and Analysis (SACEMA), as well as the Department of Botany and Zoology, Stellenbosch University for supporting and hosting this research. I would like to thank my supervisor Professor Theresa Wossler for all her support, her guidance, for letting me step out into a new field and for standing by me as I explored and researched. I would like to thank my co-supervisor professor Cang Hui, for his insightful input and for challenging me to look deeper and broader, showing me the wonderful capacity of mathematical modelling. I want to extend my thanks to Shula Johnson and Jannine Basson for their technical and administrative support. I thank the entire Wossler lab that were with me during this period but in particular would like to thank Nanike Esterhuizen for running the gauntlet with me, Natasha Mothapo for sparking my love of ants during my undergrad as well as introducing me to social immunity, and Chris du Toit, my first honours student who aided me in collecting, conducting experiments and watching videos of *C. fulvopilosus*. I want to thank the Healing Farm, Helderberg Nature Reserve and Cape Nature for allowing me to collect ants. I would like to thank Welri Nortje for her aid during analysing video data and Duncan Mc Dougall for teaching me to use code to simplify tasks and for being an ear to listen when I needed it. I would like to thank Herman Kamper for introducing me to python. I'd like to thank my family, both close and extended for their support, I'd also like to acknowledge the support of my church family that helped me to maintain a life balance during this dissertation. None of this would have been possible without the love, support and friendship of my wife. Nikki you are my rock and guiding light, you helped me up when I was low. This dissertation would not have come together without you there beside me; I love you and will never stop thanking you. Finally, I would like to thank my God for bringing all these generous people into my life and for showing me how amazing and wonderful this world is, with its constant evolution.

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Chapter 1: General Introduction

Social insects represent an extremely successful group of organisms, excelling ecologically and dominating numerically worldwide, which has largely been attributed to their social lifestyle. Permanent group living has been proposed as one of the most impactful evolutionary transitions (Smith & Szathmary, 1997), allowing for shared defence and resource acquisition between individuals. The social insects, including among others, ants, bees and termites have all evolved eusociality, a more complex system of group living. Eusociality is defined as group living organisms that engage in alloparental care (caring for the offspring of nestmates), having overlapping generations and a division of labour including reproduction (Hölldobler & Wilson, 1990). Eusociality provides competitive advantages and opens up new niches, as colonies exhibit flexibility in their allocation of workers to tasks dependent on internal and external factors (Robinson, 1992). The benefit of division of labour and alloparental care facilitates an increased density of individuals within a social group. Larger structured groups have been shown to outcompete less structured, smaller aggregations of the same species (Hölldobler & Wilson, 1990). Despite the benefits associated with increased sociality, an increased density of individuals in a colony can poses health risks. Specifically, high population densities and contact rates are expected to result in increased pathogen loads and spread (Hamilton, 1987; Schmid-Hempel, 1998; Godfrey *et al.*, 2006). This is further exacerbated by increased genetic relatedness (Luong *et al.*, 2007), resulting from the reliance on a few reproductive individuals, which increases the proportion of related individuals within a group. Despite expected health costs associated with eusociality, social insects seldom succumb to natural disease epidemics. That is not to say they are immune to epidemics as the farming of honeybees has resulted in many epidemics such as the widespread American foulbrood (Genersch, 2010) or the zombie ant fungi representing recurrent *Cordyceps* (Evans & Samson, 1984) and *Ophiocordyceps* (Andersen *et al.*, 2009; Imirzian *et al.*, 2019) infections experienced by ants in tropical environments.

Research has attributed the lack of widespread colony epidemics to the immune system of social insects (see table 1.1) that exploit a range of physiological (Schmid-Hempel, 2005) and behavioural actions (De Roode & Lefevre, 2012) at individual (Vainio *et al.*, 2004) and group levels (Ugelvig & Cremer, 2007). Individual immunity, which represents an individual's innate immune system, presents the first barrier preventing epidemics from establishing. A pathogen must first successfully infect an individual and then penetrate the nest before it can spread.

Innate immunity is the focus of on-going research (Danihlík *et al.*, 2015; Viljakainen, 2015; López-Uribe *et al.*, 2016), including gene methylation (see Yan et al. 2015 for review) and regulation of immune responses (see Wilson-Rich et al. 2009 for review). Actions undertaken by an individual for the control of pathogen spread, which benefit the colony, have been collectively termed "social immunity" (Cremer *et al.*, 2007) and are expected to provide the main line of defence in eusocial insects. Social Immunity (SI) actions are varied, complex and have seen an upsurge in research outputs (see Cremer *et al.*, 2007; Cremer & Sixt, 2009; Stroeymeyt *et al.*, 2014; Meunier, 2015; Van Meyel *et al.*, 2018; Liu *et al.*, 2019 for reviews).

Table 1.1: A subset of Insect immune system mechanisms.

Mechanism		**Brief explanation of behaviour**	**Source**
Individual immune responses	Recognition of foreign bodies	The insect immune system has developed receptors to identify potential pathogens. For example, receptors have been developed that attach to peptidoglycan or lipopolysaccharide, bacteria cell wall components, or β-1,3-Glucans, a fungal cell wall component.	(Kanost *et al.*, 1988; Ladendorff & Kanost, 1990; Ochiai *et al.*, 1992; Morishima *et al.*, 1995)
	Phagocytosis of foreign bodies	If foreign bodies are small enough, hemocytes in the insect's hemolymph will engulf the foreign bodies isolating them from the hemolymph.	(Bayne, 1990)
	Nodule formation	In the case of larger foreign bodies, many hemocytes may adhere to the surface of the foreign body thereby isolating it from the hemolymph	(Marmaras & Charalambidis, 1992; Charalambidis *et al.*, 1994, 1996; Marmaras *et al.*, 1994)
	Encapsulation	Encapsulation occurs when many layers of hemocytes surround a foreign body, which may or may not be surrounded by a melanin coat. Encapsulation can also occur in the absence of hemocytes through the production of a melanin coat alone	(Götz, 1986; Christensen & Severson, 1993)
	Anti-microbial production	When foreign bodies are detected insects can secrete anti-microbial products into their hemolymph to combat these potential infections. This has been detected for bacteria and for fungi	(Brey *et al.*, 1993; Iijima *et al.*, 1993; Lee & Brey, 1995)
Individual immunity	Immune priming	Temporary immunity to a pathogen that has been encountered previously, can be developed via active or passive immunisation	(Kurtz & Franz, 2003; Sadd & Schmid-Hempel, 2006; Roth *et al.*, 2009)
	Active Immunisation	Is the result of interaction with attenuated pathogen strains or very low doses of pathogen or in response to a signal released by infected individuals	(Konrad *et al.*, 2012)
	Passive Immunisation	Is the result of a transfer of protective immune products from one individual to another	(Hamilton *et al.*, 2011)
	Herd/ community immunity	If sufficient resistant/immune individuals are present susceptible individuals may be protected as pathogens may go locally extinct due to lack of potential hosts	(John & Samuel, 2000)

Social immune mechanisms may be prophylactic or activated (see Table 1.2). Prophylactic mechanisms include social actions or systems that, when implemented, decrease the likelihood of pathogen spread in a colony. A few examples of prophylactic behaviours include: division of labour (Naug & Camazine, 2002; Griffiths & Hughes, 2010), where individuals are allocated to tasks based on their age or caste, consequently the spread of disease is minimised as older individuals or a more expendable caste undertake tasks with increased pathogen encounter rates. Ants can also manage their use of space; this compartmentalisation can separate reproductives from potentially infected foragers or keep waste sites separate from the rest of the nest (Hart & Ratnieks, 2001). Together these traits are termed organisational immunity and result from variations in spatial use and interactions based on age and task. A study that tracked individuals within a colony of *Camponotus fellah* found that the ants separated into three distinct groups and individuals engaged more within groups than between (Mersch *et al.*, 2013). Ants modify their interactions with potentially hazardous nestmates, for example, fungus growing ants aggress nestmates that manage the refuse piles if they attempt to enter the nest or fungus garden (Hart & Ratnieks, 2001; Ballari *et al.*, 2007). Social insects collect substances with antimicrobial properties from their local environment and use these substances to sanitise the nest (Christe *et al.*, 2003; Chapuisat *et al.*, 2007; Simone-Finstrom & Spivak, 2012; Brütsch & Chapuisat, 2014). Ants engage in many hygienic behaviours, such as nest cleaning or corpse removal from the nest (Ortius-Lechner *et al.*, 2000; Hughes *et al.*, 2002; Diez *et al.*, 2014), decreasing latent pathogen threat levels, particularly when mortality has resulted from an infection.

Table 1.2: A Summary of social immune behaviours implemented by the social insects.

SI Mechanism		**Brief explanation of behaviour (A): Activated (P): prophylactic**	**Source**
Organisational immunity	Division of labour	Tasks are allocated to workers based on their age, with older individuals undertaking more dangerous tasks (P)	(Naug & Camazine, 2002; Griffiths & Hughes, 2010)
	Compartmentalisation	The spatial and temporal separation of important colony members (brood or reproductives) from those that exit the nest (P)	(Hart & Ratnieks, 2001; Mersch *et al.*, 2013; Baracchi & Cini, 2014)
	Varied interaction rates between tasks	Individuals undertaking hazardous jobs experience decreased or anti- social interaction (P)	(Hart & Ratnieks, 2001; Ballari *et al.*, 2007)
	Altruistic suicide	Infected individuals leave the nest to prevent the spread of infection (A)	(Heinze & Walter, 2010; Rueppell *et al.,* 2010)
	Antisocial behaviour when infected	Infected ants become antisocial to nestmates and brood (A)	(Bos *et al.*, 2012; Leclerc & Detrain, 2016)
Hygienic behaviours	Use of antimicrobials	Antimicrobial substances can be collected from the local environment, produced via glandular secretion for use in sanitising the nest (P)	(Chapuisat *et al.*, 2007; Simone-Finstrom & Spivak, 2012; Brütsch & Chapuisat, 2014)
	Use of antifungal faecal pellets	In termites faecal pellets show strong antifungal properties and are used in nest construction to prevent pathogen growth (P)	(Rosengaus *et al.,* 1998)
	Corpse removal	Corpses are removed from the nest in order to prevent secondary infections occurring (P)	(Ortius-Lechner *et al.*, 2000; Hughes *et al.*, 2002; Diez *et al.*, 2014)
	Corpse burial	Infected corpses are buried to prevent secondary infections from occurring, buried corpses can also be covered with fecal matter (P/A)	(Chouvenc & Su, 2012)
	Cannibalism of infected corpses	If infection rates are low colonies can cannibalise infected corpses to break down and stop potential secondary infections (P/A)	(Chouvenc & Su, 2012; Rosengaus & Traniello, 2001)
	Allogrooming	Individuals will groom each other to decrease their external pathogen load and lessen the chance of subsequent infection (A)	(Okuno *et al.*, 2012; Zhukovskaya *et al.*, 2013; Theis *et al.*, 2015)
	Immune priming	Individuals can, by sharing a low infection load or by the potential sharing of immune substances, potentially impart a temporary immunity to the pathogen in question (A)	(Traniello *et al.*, 2002)
	Pathogen removal/ inactivation in nest	If a pathogen is detected in the nest it is sanitised and removed from the nest (A)	(Jaccoud *et al.*, 1999)
	Removal of infected brood	If brood is infected with a lethal pathogen they are removed from the nest (A)	(Ugelvig *et al.*, 2010)
	Social fever	Nestmates will gather and raise their body temperature and that of their surrounds in	(Starks *et al.*, 2000)

		order to raise the ambient temperature and kill the pathogen (A)	
Colony behaviours	Nest abandonment	If a pathogen has overrun the nest and hygienic behaviours cannot arrest it's spread, then a colony will abandon its nest (A)	(Drees *et al.*, 1992)
	Infection alarms	In some social insects infected individuals will engage in alarm behaviour to warn nestmates of their status (A)	(Rosengaus *et al.*, 1999; Myles, 2002)

Activated responses are employed when exposed to pathogens or if an infection is detected. An important activated response is the mechanical removal of pathogen conidia from nestmate cuticles via allogrooming (Okuno *et al.*, 2012; Zhukovskaya *et al.*, 2013; Theis *et al.*, 2015). Allogrooming increases the likelihood of survival of the exposed individual by decreasing their cuticular pathogen load. The implementation of allogrooming deactivates the ingested pathogens as a result of antibiotic compounds maintained in the infra-buccal pouch (Little *et al.*, 2006), which acts as a reservoir for indigestible solid particles which are stored and later expelled as pellets (Eisner & Happ, 1962). During allogrooming, ants will also apply glandular secretions which have antimicrobial properties, to further hinder and prevent infections (North *et al.*, 1997; Fernández-Marín *et al.*, 2006; Tragust *et al.*, 2013). Furthermore, it has been suggested that allogrooming may facilitate immune priming (Traniello *et al.*, 2002; Konrad *et al.*, 2012; Rosengaus *et al.*, 2013; Masri & Cremer, 2014), the insect equivalent of vaccinations; however, this is still under debate (Reber & Chapuisat, 2012b). Alternate activated responses include attempts to deactivate or rid the nest of infectious particles, as seen in the ant *Atta sexdens rubropilosa* where individuals will attempt to collect and cover fungal conidia even at the risk of a lethal infection to protect the colony (Jaccoud *et al.*, 1999). Ants, namely *Cardiocondyla obscurior,* will also remove *Metarhizium anisopliae* infected brood (Ugelvig et al. 2010). Furthermore, ants have been shown to destructively disinfect pathogen exposed pupae (Pull *et al.*, 2018). These behaviours are similar to bees uncapping and removing infected larvae (Boecking & Spivak, 1999; Spivak & Reuter, 2001). Ants may even cut up corpses and expose them to sunlight (Reber & Chapuisat, 2012a); this mutilation speeds up dehydration whilst the UV light deactivates conidia (Fernandes *et al.*, 2007), decreasing the likelihood of pathogen spread. A recent study undertaken by Diez et al. (2015) showed that members of *Myrmica rubra* colonies responded differently to fungus-killed ants as opposed to freeze-killed ants. The ants increased their grooming rates and moved fungus-killed corpses further away, suggesting that ants can identify pathogen risks and modify behaviour accordingly. Infected

ants may also either leave the nest, committing altruistic suicide, or they may decrease their contact rates with colony members when infected (Heinze & Walter, 2010; Bos *et al.*, 2012; Leclerc & Detrain, 2016), which may decrease the likelihood of infections but also decreases the work load for corpse removal. Alternatively, colony size may play a role in disease defence, with smaller colonies taking longer to remove infectious conidia from the nest than larger colonies (Leclerc & Detrain, 2018). In the rare case that an epidemic becomes unmanageable ants may even abandon their nests and relocate to a new site to escape infections (Drees *et al.*, 1992); yet, this is more likely to occur in smaller colonies (Leclerc & Detrain, 2018).

Pathogen risks do not occur uniformly across social insects, as pathogens have evolved to infect most social insects, which vary in ecology and life history. Boomsma and colleagues (2005) reviewed parasite pressures across the major groups of social insects. They showed that bees and wasps are expected to suffer from orally transmitted diseases, given their use of shared resources (i.e. pollen resources) and are also more likely to be affected by macroparasites, particularly in wasps (due to their predatory nature). Ants and termites, given their soil-based nesting ecology, are expected to encounter nematodes, helminths and fungi (Boomsma *et al.*, 2005). Fungal pathogens are particularly prevalent in and around the nests of ants (Reber & Chapuisat, 2012a; Araújo *et al.*, 2018) and do infect ants. Fungal pathogens infecting ants are generally grouped into two classes, specialist fungi and generalist fungi.

Specialist fungi such as those from the genus *Ophiocordyceps* (Araújo *et al.*, 2018), infect their host and manipulate the behaviour of their host. They force their host to climb branches and bite down on leaf veins before killing their host (Andersen *et al.*, 2009; Pontoppidan *et al.*, 2009), then sporulate releasing conidia or ascospores on the surfaces below. These specialist fungi develop extremely close associations with their hosts, maintaining a consistent low-level infection pressure, with a study finding a consistent infection affecting all colonies in the study area for the entire 20-month assessment period (Loreto *et al.*, 2014). In contrast to the specialist fungi, the generalist fungi, specifically entomopathogenic fungi, will infect a broad range of hosts and after proliferating in their hosts will kill them and sporulate from their cadaver. Ants and termites are not exempt from generalist fungal infections, such as *Metarhizium anisopliae* and *Beauveria bassiana*, with studies isolating these fungi from the soil and ants within colonies (Hughes *et al.*, 2004; Reber & Chapuisat, 2012a). These generalist fungi are extensively used to assess social immunity behaviours (Traniello *et al.*, 2002; Hughes & Boomsma, 2004; Pie *et al.*, 2005; Schmidt *et al.*, 2011; Purcell *et al.*, 2012; Ho & Frederickson,

2014; Tranter & Hughes, 2015; Qiu *et al.*, 2016), due to their ease of collection, maintenance and non-specific pathogenicity. More recently, however, the ecological significance of using a generalist pathogen to assess colony responses to infection has been questioned (Loreto & Hughes, 2016). Regardless of specificity, most fungal pathogens release conidia en masse into the environment and wait for an encounter with a potential host. Once conidia find a host they will attempt to germinate and pierce the host's cuticle, a process that can take longer than 24 hours to occur (Vestergaard *et al.*, 1999). This provides potential hosts a small window during which they can remove conidia from their cuticle before an infection occurs. Many social immunity mechanisms capitalise on this window of opportunity. Social immune mechanisms in an ant species are selected by those pathogens most frequently encountered, as such, temperate species are unlikely to have evolved defences against *Ophiocordyceps* which occur primarily in tropical forests (Araújo *et al.*, 2018).

The ecological niche of a species, as well as life histories, will influence the social immune mechanisms in which a particular species invests. For example, soil nesting ant species are expected to encounter more pathogens than arboreal nesting ants. An assessment of the variation in disease defences across seven neotropical ants depicting an arboreal or soil nesting structure, showed that soil nesting ants did not invest more in disease defence than arboreal ants (Walker & Hughes, 2011). Ants that nested arboreally, but foraged in both arboreal and soil environments, showed the greatest resistance, as these species likely encountered a wider range of pathogens (Walker & Hughes, 2011). In a more recent study, the capacity for species to detect and react to pathogens was assessed in four species with varied life histories: Weaver ant, wood ant, leaf-cutting ant and a harvester ant; they found that all of the species were able to detect and respond to pathogen exposure (Tranter *et al.*, 2014). Yet, species invested differentially in a range of control behaviours, with weaver ants self-grooming to a greater degree and leaf-cutting ants investing more in allogrooming to mitigate pathogen exposure (Tranter *et al.*, 2014). Disease defence mechanisms in the fungus farming ants, which reflect similar life histories, have revealed that the more derived the species the greater their reliance on metapleural gland secretions for pathogen defence (Tranter et al., 2015). They showed this by experimentally blocking these glands and found an increased mortality in the derived fungus farming ants when compared to basal fungus farming ants (Tranter et al., 2015). In the absence of a metapleural gland ants have to rely on other forms of pathogen defence; the weaver ant *Oecophylla smargdina*, has been shown to rely on potent venom secretions for the control of pathogens and revealed lower rates of grooming than four species of *Polyrhachis* weaver ants

which all responded similarly to pathogen exposure (Tranter & Hughes, 2015). In a more recent study by Bos and colleagues (2019), the susceptibility of 12 naturally co-occurring ant species to infection by two generalist fungal pathogens was assessed. They found pronounced differences across the tested species and pathogens, with differences in susceptibility occurring even within genera. Furthermore, ants with a greater susceptibility to pathogens showed overall greater rates of allogrooming than those that were less susceptible (Bos *et al.*, 2019).

Despite a growing number of studies assessing social immune mechanisms across species there remains a geographic bias in these studies with most studies occurring in the northern hemisphere or in tropical regions. In particular their remains a distinct gap in the assessment of social immunity mechanism in African ants (but see Frank *et al.*, 2018). As such this thesis sought to assess social immunity mechanisms in three South African ant species, *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre*. In South Africa there is a substantial paucity in assessments of ant ecology as such not much is reported regarding nest structure, reproductive ecology, degree of melanisation and feeding habits, we have included information on the species based on personal observations and the little literature that exists on our species of interest. *Anoplolepis custodiens* represents an ecologically dominant ant species in Southern Africa (Samways, 1990), that occurs primarily in semi-arid environments (see Figure 1.1 A). They are characterised by extremely aggressive behaviour and can even become pest species in agricultural settings particularly when they have access to aphid mutualists (Addison & Samways, 2000). Foragers recruit en masse to resources and have even been shown to attack and eliminate colonies of the social spider *Stegodyphus dumicola* (Keiser *et al.*, 2015). They can build large interconnected nests with multiple queens, further, their workers are polymorphic and range in size from 4 mm to 8 mm in length. *Camponotus fulvopilosus* represents an arid-adapted species with a distribution across the arid regions of southern Africa (See Figure 1.1 B). Colonies form claustrally and occur in sandy soils but given time are able to grow into very large colonies (Robertson & Zachariades, 1997). Workers are polymorphic with both minors and majors present in mature nests, *C. fulvopilosus* ants can grow to very large sizes with majors reaching 18 mm in length. *Camponotus fulvopilosus* workers generally forage alone and they are well known for their excellent eyesight reacting and tracking movements of observers, further they have been observed to spray formic acid up to 15 cm in defence of the colony (Robertson & Zachariades, 1997). *Tetramorium sericeiventre* occur throughout Africa across a wide range of environmental clines (Figure 1.1 C), they are an opportunistic ant species that are able to coexist with a range of species including the invasive

and extremely aggressive Argentine ant (Luruli, 2007). They have relatively small nests of a few hundred individuals with one or more queens (personal observations), occurring in moist soils and are expected to encounter more soilborne fungal pathogens given their moist environment. Workers are monomorphic and are generally small reaching around 4 mm in length. We have chosen to assess social immune mechanisms in these species, despite the lack of phylogenetic relatedness as these three South African ant species represent common ant species with distinct life histories and social organisations.

By taking a multispecies approach we are able to gain broader insights into the implementation of social immunity by assessing its use and importance across life histories and social organisations. Ant species have been shown to invest in a range of social immunity mechanisms for pathogen control that are expected to differ based on the ecology of the pathogen. Specifically, in fungal infections, ants have a window of opportunity to remove conidia from their cuticle prior to the germination of the conidia. Our hope was that a multispecies and holistic approach to assessing social immunity in South African ants would provide much needed knowledge at a broad scale, instead of implementing a focused and detailed assessment of a single species. Given the paucity of social immunity studies from a warm temperate African perspective and the potential window of opportunity for conidia removal prior to germination, this study will be the first assessment of social immunity mechanisms in three South African ant species. We assessed how ants managed exposure to a generalist entomopathogenic fungus, specifically during the initial time frame after infection when conidia attachment is low. We assessed a range of social immune mechanisms through multiple approaches, considering direct conidia control mechanisms as well as changes in interaction and spatial use patterns. This thesis is structured as a collection of papers and as such there is a degree of overlap in content to ensure that each can stand alone. The pronoun “we” is used throughout the thesis to acknowledge the input of my supervisors, but I declare that this dissertation is entirely my own work.

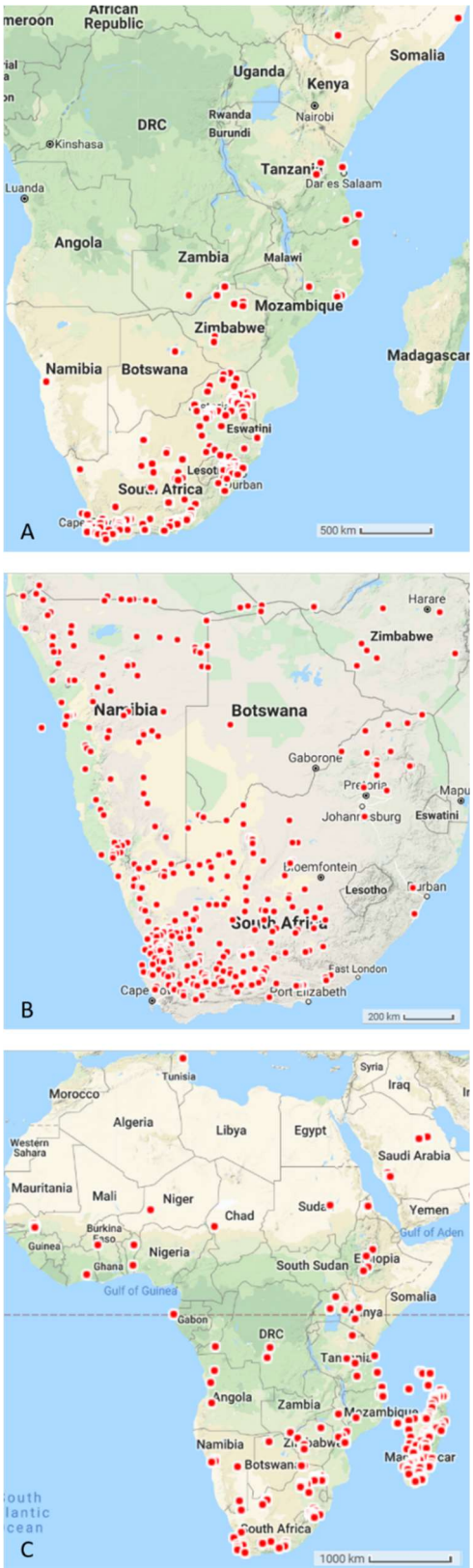

Figure 1.1: Species distribution records in Africa and surrounds for A: *Anoplolepis custodiens*, B: *Camponotus fulvopilosus* and C: *Tetramorium sericeiventre*. Reproduced from http://www.antweb.org.

This thesis consists of five data chapters and a general discussion (Chapter 7). In Chapter two we assess how the three species, *A. custodiens, C. fulvopilosus* and *T. sericeiventre* respond to infection as group size increases. Specifically, we assessed the frequency and duration of trophallaxis, self- and allogrooming a treated-individual experiences when exposed to increasing group sizes. We further assessed survival across the species and treatments for both the treated individual and their nestmates. Finally, this chapter assessed the effects of conidia reduction in the three species when individuals have access to self-grooming, allogrooming and secondary transfer.

In Chapter three we assessed how over time, experimental colonies of the three species altered their investment into allogrooming and trophallaxis when treated with a control substance or a pathogen. Specifically, this was accomplished by recording intact colonies and exposing them to a control and pathogen and tracking the frequency and duration of all trophallaxis and allogrooming events.

In Chapter four we used the same experimental setup as Chapter three in order to assess how the three species utilise the available nest space and how they alter this in response to infection. Specifically, we assessed spatial use patterns, degree of clustering and distance of individuals to the queen in the three species under control and pathogen exposures.

Chapter five focused specifically on *C. fulvopilosus* to assess how interaction networks shift under control and pathogen exposure treatments. Specifically using our previous recoding setup and individually marked individuals we tracked interactions and built interaction networks under control and pathogen treatments. We evaluated the overall connectivity and structuring of the networks and assessed shifts under different treatments.

In Chapter six, the data collected in Chapter 2 and 3 are used to build matrix projection models, which predict conidia removal rates for each species. These models were compared to assess how each species managed pathogen exposure. Thereafter the models were altered to knock out either self-grooming or allogrooming to assess their importance to social immunity for each of the species. Finally, we altered the initial exposure dose within the models to assess whether a greater pathogen load affected conidia removal rates in each of the species.

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Chapter 2: Density-dependent sanitary behaviours for effective entomopathogenic fungi control in three African ant genera

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Abstract:

Generalist entomopathogenic fungi occur around colonies of ants and pose a risk to colonies, which are characterised by nests of densely populated, highly related individuals. Ants combat these threats via social immunity mechanisms like allogrooming, self-grooming and trophallaxis to remove and inactivate pathogens. Ant species should differ in social immunity mechanisms based on ecology, life history and colony dynamics. This study examined how three South African ant species, *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre* manage pathogen exposure across increasing group sizes. We assessed survival of ants exposed to a generalist fungal pathogen, *Metarhizium anisopliae*, and assessed the frequency and duration of self-grooming, allogrooming and trophallaxis relative to an infected ant across increasing group sizes. Furthermore, we evaluated the efficacy of self-grooming and allogrooming on conidia removal in each species. Survival did not differ between pathogen and control exposure for all three species, implying effective mitigation of pathogen exposure. Species increased the frequency and duration of immune mechanisms when in larger groups and on day one following exposure, furthermore they relied primarily on self-grooming. Self-grooming reduced conidia loads most in each species, followed by allogrooming for *A. custodiens* and *T. sericeiventre*; however, *C. fulvopilosus* did not benefit from allogrooming. Our study showed that species can modify interaction frequencies and duration in response to increased group size to limit pathogen threats and that not all species utilise the same mechanisms to mitigate pathogen exposure.

Intended for submission to "Behavioural Ecology" or "Behavioral Ecology and Sociobiology"

Introduction

Microorganisms are ubiquitous within nature and can be beneficial, harmless or harmful, and these harmful microorganisms can take the form of pathogens and parasites. During foraging bouts, terrestrial workers of social insects are frequently exposed to pathogens and parasites, including entomopathogens. An entomopathogenic fungal infection requires the identification of a suitable host, followed by attachment and then penetration of the host insect's cuticle. The fungus evades the immune function of its host (Huxham *et al.*, 1989), proliferates and produces toxins (Vey *et al.*, 2002) eventually causing the host's death, after which the fungus emerges from the cadaver and sporulates, to infect nearby potential hosts (Castrillo *et al.*, 2005). Entomopathogenic fungi (EPFs) can represent both generalist and specialist pathogens of arthropods (Hajek and Leger 1994, Meyling and Eilenberg 2007 and references within), with high levels of EPFs previously isolated from the environments surrounding social insect colonies (Milner *et al.*, 1998; Schmid-Hempel, 1998; Hughes *et al.*, 2004; Reber & Chapuisat, 2012). Excavating and foraging workers in terrestrial social insects are predicted to frequently encounter EPF conidia or ascospores resting in the soil or may come into contact with EPFs from sporulating cadavers (Meyling & Eilenberg, 2007) of other insects. Moreover, *Ophiocordyceps* infected ants are manipulated by the fungus to attach to branches near foraging paths where they sporulate, infecting other passing individuals (Andersen et al., 2009; Pontoppidan et al., 2009; Hughes et al., 2011). Entomopathogenic fungi have been isolated directly from field collected ants (Reber *et al.*, 2008) and subsequently as exposed workers reintegrate with related colony members, in any social insect system, the pathogens presumably spread.

The high density of closely interacting individuals resulting from social grouping are expected to increase susceptibility to these pathogens (Schmid-Hempel, 1998). Secondary entomopathogen infections, when conidia are transferred from the initial host to another individual, occur through body contact prior to the conidia penetrating the host's integument (Konrad *et al.*, 2012). Thus, the occurrence of secondary infections is expected to be high; however, this is not always the case. Social insects employ a suite of behaviours above and beyond their individual immunity to combat the spread of infection; these behaviours benefit the colony and are thus termed social immunity (SI: Cremer *et al.*, 2007). Among the social immune behaviours performed to counter secondary entomopathogenic fungal infections is the implementation of prophylactic (preventative) and activated measures, such as self-grooming, allogrooming or trophallaxis, hereafter collectively termed sanitary behaviours.

Allogrooming is the process whereby individuals clean the cuticle of their nestmates, removing pathogens and parasites (Loreto *et al.*, 2014). Allogrooming decreases the conidia load on the infected nest-mate, during the period when conidia attachment strength is low (Vestergaard *et al.*, 1999). The removal of conidia decreases the risk of the exposed individual succumbing to the infection; however, this allogrooming also runs the risk of spreading the infection to the grooming nestmate, albeit at a vastly decreased chance (Hughes *et al.*, 2002). Ants are expected to increase their rate of self-grooming in response to external pathogens such as entomopathogens (Reber *et al.*, 2011; Okuno *et al.*, 2012). With regards to allogrooming frequency and intensity in ants, contradictory findings show both increased allogrooming towards infected nest-mates in some cases (Hughes *et al.*, 2002; Walker & Hughes, 2009; Bos *et al.*, 2012; Okuno *et al.*, 2012) and no increase in allogrooming in other cases (Graystock & Hughes, 2011; Reber *et al.*, 2011; Theis *et al.*, 2015). Mathematical models assessing the efficacy of immunity, nest hygiene and allogrooming have shown that increased allogrooming is favoured under periodic infections but costly when under constant threat from pathogen infection (Fefferman *et al.*, 2007). Only a single study has reported a decrease in allogrooming rates; although, this decrease was from the infected individual towards nestmates (Theis *et al.*, 2015), suggesting that allogrooming is a highly effective control method which is either maintained at constant level as a prophylactic immune action or increased in the face of infectious agents (Diez *et al.*, 2015). This variation in reliance on allogrooming across species and systems may be due to the costs involved in mounting the response. Whilst receivers of allogrooming benefit from decreased conidia loads the donor risks secondary infection therefore, allogrooming may be under varying selective pressures depending on infection risks, population densities and species innate immunities. Rates of sanitary grooming may vary and are predicted to increase in larger colonies (Schmid-Hempel, 1998) and under higher pathogen loads. This theoretical relationship between the rate of allogrooming, pathogen load and local density of nestmates reveals that intense grooming is favoured at higher group sizes and at intermediate pathogen loads (Figure 2.1).

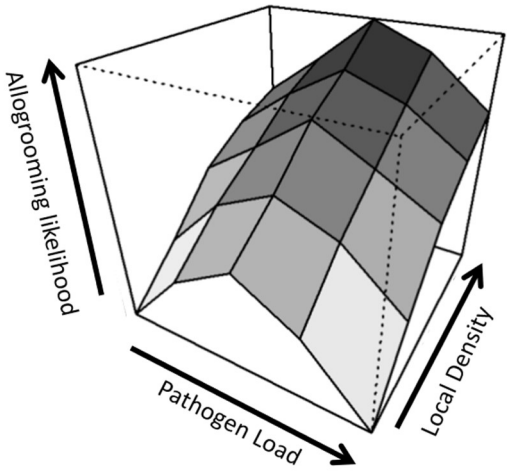

Figure 2.1: Theorised relationship between the rate of allogrooming, pathogen load and the local density of nestmates. Darker colours represent higher likelihoods of allogrooming occurring.

Self-grooming is expected to be an ant's primary mechanism for the control of surface infections and infected ants frequently upregulate self-grooming in response to infections (Bos *et al.*, 2012; Tranter *et al.*, 2014; Diez *et al.*, 2015; Theis *et al.*, 2015). Self-grooming combined with allogrooming is presumed to control most surface infections. An individual's health status is likely conveyed through the exchange of regurgitated substances via trophallaxis or changes in cuticular hydrocarbons (Richard et al., 2012; Pull et al., 2018). Thus, interactions such as allogrooming and trophallaxis are fundamental in communicating pathogen status, with trophallaxis even providing a source of antimicrobial substances. In *Camponotus fella* it was shown that immune challenged individuals donated trophalactically more than naïve individuals and that the regurgitated droplets from immune challenged individuals showed greater antimicrobial activity (Hamilton *et al.*, 2011). The potential benefits of trophallactic exchange in a SI context must be weighed against the potential risk it represents by facilitating pathogen transmission. It is important to assess trophallaxis under immune challenges since a colony must trade-off mitigating disease spread and ensuring sufficient flow of resources (Blonder & Dornhaus, 2011), especially as colony size increases.

Given that social insects frequently experience dense nest populations, costly disease control mechanisms might be density dependent. An example of this is the theorised density dependent prophylaxis (DDP) hypothesis which predicts an upregulation in disease defences under dense population conditions (Wilson & Reeson, 1998). Analyses of DDP in social insects have been assessed with varying results, DDP has been observed in bumblebees (Ruiz-Gonzalez *et al.*, 2009) and ants (Hughes *et al.*, 2002). Most studies assessing DDP assess individual immunity and do not account for possible changes in sanitary behaviours. Overall encounter rates are expected to increase with larger colony size (Gordon *et al.*, 1993; Thomas & Elgar, 2003; Holbrook *et al.*, 2011); however, changes in per capita activity rates remain uncertain. Allogrooming mechanically removes conidia or ascospores from the cuticle, the success of which may be rate dependent, with sufficient grooming required to remove an adequate number of conidia or ascospores to overcome the pathogen challenge. If the frequency of allogrooming increases, the time taken to reach the same degree of pathogen control should be decreased, suggesting a trade-off through which variation in control strategies may arise. Although colony size and sanitary behaviour are expected to be linked, the effect of local habitat and ecological niche may also play a role in investment in sanitary behaviours.

Ants inhabit many ecological niches and consequently associate with a range of different pathogens. Hence, depending on the habitat and pathogens most often encountered, ants most likely differ in their reliance on SI mechanisms for the control of frequently encountered pathogens. The fungus growing ants, for example, readily use metapleural gland (MG) secretions in response to infections as well as grooming fungal gardens with MG secretions (Fernández-Marín *et al.*, 2006). They also display complex waste management and socially exclude risky individuals such as garbage workers (Hart & Ratnieks, 2002). In contrast, weaver ants make use of alternate strategies to mitigate their lack of MG secretions, with *Oecophylla smaragdina* relying on venom gland secretions while *Polyrhachis* species relied more on increased grooming rates (Tranter & Hughes, 2015). A recent study compared pathogen detection in four species of ants (leaf-cutting ants, harvesters, wood ants and weaver ants) with similar colony sizes but different life histories and nest architecture (Tranter *et al.*, 2014). All four species were able to detect fungi, but differed in their responses, which the authors attributed to life history differences. All species avoided fungus infected surfaces and upregulated grooming in response to infected nestmates; however, weaver ants relied on self-grooming whilst leaf-cutting ants primarily invested in allogrooming. Given this variation in

pathogen control mechanisms (self-versus allogrooming), it is therefore important to assess aspects of pathogen control across species with varied life histories and environments.

In this study we assess the efficacy and investment in sanitary behaviours in three African ant species: *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre. Anoplolepis custodiens* represents an ecologically dominant native ant species (Samways, 1990; Addison & Samways, 2000) which occurs in semi-arid environments. They are extremely aggressive, with large colony sizes and they represent the dominant Dolichoderinae functional group as per Hoffmann & Andersen (2003). *Camponotus fulvopilosus* represents an arid adapted species with a wide distribution. Colonies of *C. fulvipilosus* are formed claustrally but can grow to large sizes given time. The species occurs primarily in high sandy soils and are known to spray formic acid up to 15 cm through the air to defend the nest (Robertson & Zachariades, 1997). *Camponotus fulvopilosus* can be classified as a subordinate Camponotini (Hoffmann & Andersen, 2003). Finally, *T. sericeiventre* represents an opportunist ant species that has been shown to coexist with many species including invasive species such as the Argentine ant (Hoffmann & Andersen, 2003; Luruli, 2007). *Tetramorium sericeiventre* has a wide distribution across Africa and across environmental clines. Not only do these species inhabit different environments but also encompass different life histories, such as solitary versus coordinated foraging, varying group sizes and polydomous versus monodomous colonies, which could result in different investment in sanitary behaviours.

Temporal variation in the investment in sanitary behaviours, particularly self-grooming and allogrooming, should be upregulated during the 24-48-hour window of opportunity, after infection, when conidia attachment strengths are weakest (Vestergaard *et al.*, 1999). This study assesses variation in the efficacy and investment of sanitary behaviours during this window of opportunity. Specifically, we assess whether pathogen challenge leads to increases in self-grooming, allogrooming or trophallaxis and prevents infection in three African ant species. Further we assess whether these changes react in a density dependent manner and assess the efficacy of grooming behaviours at reducing conidia loads in the three species. We expect to find an increase in the investment into sanitary behaviours, potentially through increased frequency of allogrooming for shorter periods in response to infection, alternatively allogrooming may occur for longer but less frequently. We expect these shifts may offset the expected increase in mortality resulting from pathogen challenge (Theis *et al.*, 2015), by limiting secondary transfer opportunities. We expect that larger group sizes will invest more in

sanitary behaviours, to offset the risks of increased density (Schmid-Hempel, 1998) and finally we expect that *A. custodiens* should invest most heavily in sanitary behaviours given their ecological dominance and large colony sizes. In contrast, *C. fulvopilosus* should invest the least given their adaptations to xeric environments and frequent use of formic acid.

Methods

Experimental setup

Collections and Maintenance

Ten colonies each were collected for *A. custodiens* from within the Helderberg Nature Reserve (34° 03' 43.5" S, 18° 52' 24.6" E), *T. sericeiventre* from Jan Marais Park (33° 55' 59.3" S, 18° 52' 23.0 "E) and *C. fulvopilosus* from a private farm in Montagu (Healing Farm, 33° 38' 28.5" S, 20° 02' 57.7" E). All collected colonies were maintained in plastic nesting boxes (32 X 21.5 X 7.5cm) as stock colonies, within the Department of Botany and Zoology, Stellenbosch University. Stock colonies were provided sugar water and plain water ad libitum. They were further provided weekly with termites as a protein source. All stock colonies were maintained in a temperature-controlled lab (±25 ºC) with a constant day night cycle of 12h:12h.

The entomopathogen, *Metarhizum anisopliae* (META69, isolate: ICIPE69 originally isolated from soil in the Democratic Republic of Congo; Niassy *et al.* 2012) was used in all experiments since it represents a generalist pathogen of many insect species including terrestrial ants. Owing to its wide range of hosts, *M. anisopliae* is not expected to have co-evolved with any particular ant species, but ants are expected to have evolved general defences against the pathogen given its ubiquitous nature in soils. Furthermore, it is frequently employed in SI studies (Traniello et al., 2002; Hughes & Boomsma, 2004a; Pie et al., 2005; Reber & Chapuisat, 2012; Tragust et al., 2013b; Tranter et al., 2015; Qiu et al., 2016). Entomopathogenic fungi were cultivated and maintained on Sabouraud Dextrose Agar (SDA) supplemented with 1% penicillin-streptomycin following standard procedures. We followed the procedures for fungal growth and maintenance as set out by Lacey (2012). Prior to experiments, ants were sprayed with a solution of fungal conidia grown from the META 69 oil suspensions. A spray application was utilised in order to provide an even distribution of conidia on treated ants, further, this also facilitated a size corrected dose with larger individuals receiving a larger number of conidia. This was an important consideration given the polymorphic nature of two of the three assessed species. When treated, individuals were anaesthetised via chilling, to reduce stress and ensure an even application, before being placed in a petri dish and sprayed using four pumps of a fine

mister. These ants were maintained for 24 hours before washing the conidia from the host and culturing these conidia to generate new fungal conidia. This protocol was undertaken prior to experimentation in order to maximise and control entomopathogen virulence, as maintenance on agar plates has been shown to decrease virulence (Lacey, 2012). Suspensions of conidia were made by adding scrapings of the fungal matt to a 0.05% Tween solution. The solution was vortexed and conidia concentrations assessed using a Hemocytometer. Treatment concentrations were standardised at a conidia concentration of $\pm 1.5 \times 10^7$ conidia per millilitre suspension as is frequently employed in ant studies using generalist entomopathogens (Storey *et al.*, 1991; Vergeer *et al.*, 2003; Hughes *et al.*, 2004; Reber *et al.*, 2011; Schmidt *et al.*, 2011; Purcell *et al.*, 2012; Brütsch & Chapuisat, 2014; Ho & Frederickson, 2014; Tranter *et al.*, 2015; Qiu *et al.*, 2016). We tested this concentration on the three species and found that, although mortality rates differed between species (*A. custodiens*: 85%, *C. fulvopilosus:* 83% and *T. sericeiventre:* 50%), only ~9% of all dead ants in these preliminary trials did not succumb to *Metarhizum anisopliae* infections

Local density experimental procedure

To assess the possible effects of infection and local population density on mortality and sanitary behaviour, ants were sprayed, as per previous protocols described above, with a control 0.05% Tween20 solution or infected with *M. anisopliae* (± 1.5 X 10^7 conidia per ml of 0.05% Tween20 solution). Following a modified protocol based on Hughes et al. (2002), treated ants were marked (POSCA PC-5 marker) on their abdomen for the purpose of identification. Local density was established by introducing each marked (hereafter focal) individual to either one (pair), five (small), ten (medium) or 25 (large) nestmates and maintained in a petri dish with a moist cotton ball for humidity (Figure 2.2). Individuals were allowed to interact for 48 hours after which the focal individual was separated from nestmates. Ants were maintained separately and provided food and water ad libitum. Ten five-minute recordings were conducted during the initial 48 hours, during which the frequencies and durations of all interactions with the focal individual were quantified. These interactions included allogrooming provided, allogrooming received, self-grooming and trophallaxis. Trophallaxis was defined as mouth to mouth interaction which persisted for longer than three seconds, allogrooming interactions where defined as interactions where the provider groomed the receiver for longer than three seconds and finally self-grooming was defined as an individual undertaking grooming of their own cuticle which persisted for longer than three seconds. Mortality was assessed over 14 days, cadavers (including non-focal individuals) were maintained for a further 21 days and assessed

for sporulation to determine whether mortality was a result of infection. This was repeated 10 times per species and group size with each replicate representing separate colonies.

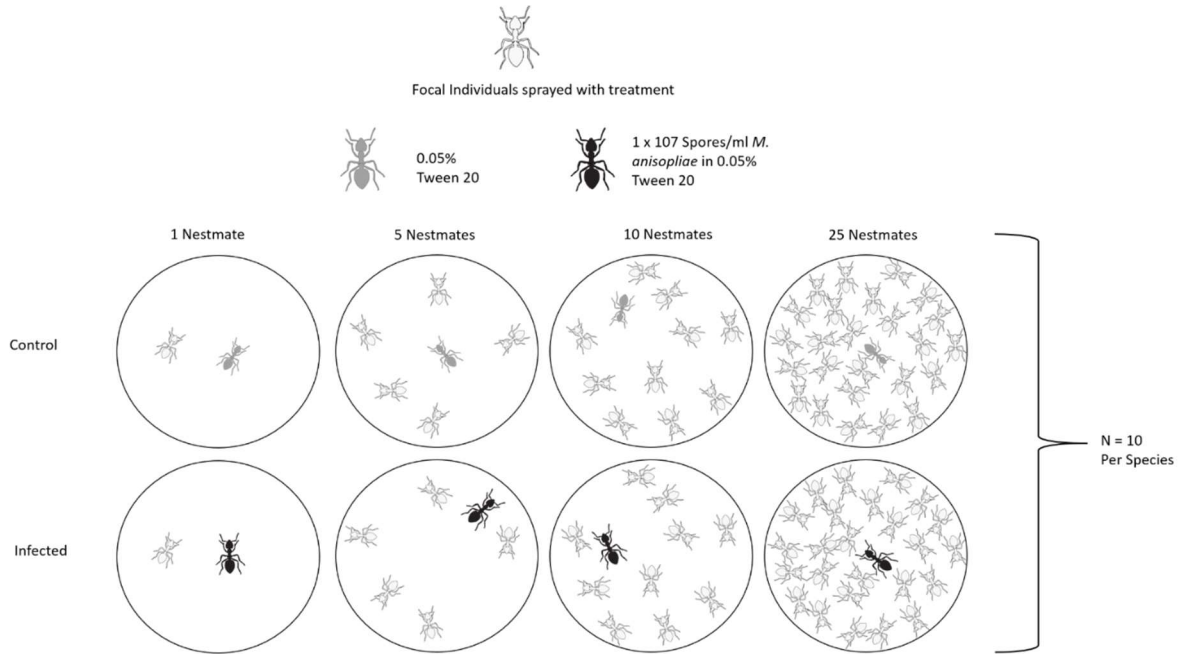

Figure 2.2: Experimental design for assessment of sanitary behaviour performed towards a focal ant treated with a control substance or exposed to a fungal pathogen. The protocol was applied in three ant species (*Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre*), assessing the effects of treatment (Tween 20: Grey ant and *Metarhizium anisopliae*: Black ant) and local density (Group sizes of 2,6,11 and 26). Untreated ants are represented as clear outlines.

Efficacy of conidia control experimental procedure

The efficacy of conidia control and potential conidia transmission between individuals were tested following a modified protocol based on the study by Tragust et al. (2013a). Thirty focal individuals per colony for *A. custodiens, T. sericeiventre* and *C. fulvopilosus* were removed from the stock colonies (n = 10 colonies/species), and 10 from each species were anesthetised via chilling, for 30-60 seconds at -18 °C. Thereafter all 30 were sprayed, as per protocol discussed above, with a 0.05% Tween fungal conidia solution (conidia solution ±1.5 x 10^7 conidia per ml). Focal ants were placed into petri dishes for one of three treatments, ants were either maintained alone (dead (n = 10) or alive (n = 10)) or placed with a naïve nestmate (n = 10) for 24 hours (Figure 2.3). After 24 hours all individuals were washed by vortexing them for 1 minute at 3000 rpm in 1ml of a 0.05% Tween solution, removing conidia from their body. From each suspension, three plates were inoculated by spreading 100 µl of the suspension on SDA agar plates supplemented with 1% penicillin-streptomycin and incubated at 23°C for 72 h, representing three technical replicates per suspension. The Colony Forming Units (CFUs) grown on each plate over this period were marked, photographed and quantified. Mean counts

of the viable CFUs from the three technical replicates were determined per treatment (dead, self-grooming, allogrooming and nestmate).

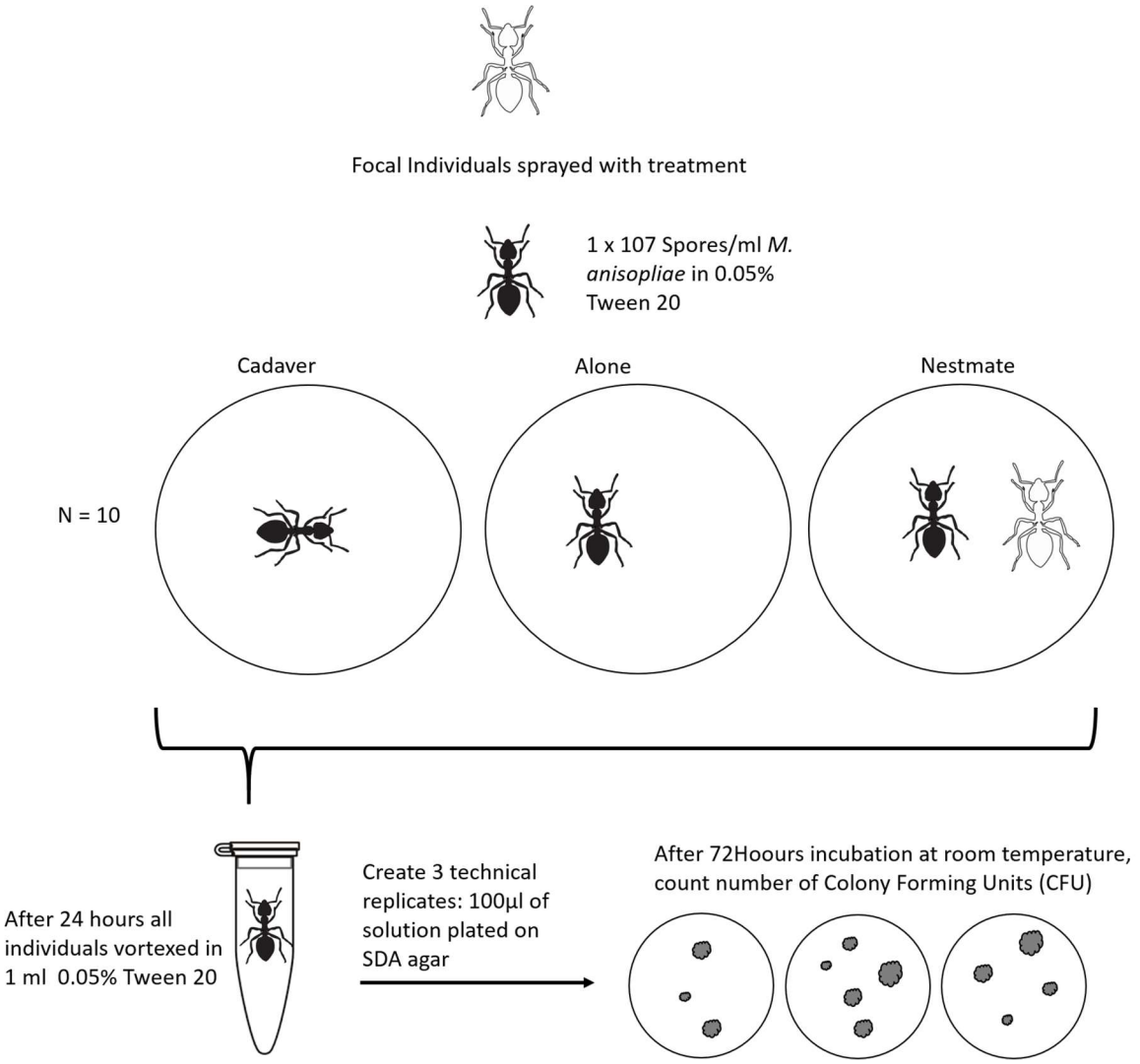

Figure 2.3: Experimental design to determine efficacy of sanitary behaviours on decreasing conidia loads in ants exposed to *Metarhizum anisopliae.* Exposed ants were kept for 24 hours under treatments with: No access to sanitary behaviours (Cadaver), access to self-grooming (Alone), access to allogrooming (treated individual) and quantifying secondary transfer (naïve nestmate). After 24 hours all ants were vortexed in Tween 20, from which three technical replicates were plated to assess the number of viable Colony forming Units (CFU) that germinated within 72 hours. This protocol was repeated for all three ant species; *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre*.

Statistical analysis

All statistical analyses were undertaken in the R statistical environment (R-Core-Team, 2013). In order to compare the effects of treatments, group size and species identity on survival, a cox-proportional hazard model was run in the "Survival" package on the focal individuals. A separate cox-proportional hazard model was run using non-focal nestmates following the same procedure. In order to determine the effect of local density on the frequency and duration of trophallaxis, self-grooming and allogrooming relative to the focal individual, we ran a GLMM using the package "GLMMTmb" (Nielsen *et al.*, 2017). We applied a negative binomial family using treatment (control or exposure), day of observation (1 or 2), behaviour (allogrooming provided, allogrooming received, self-grooming or trophallaxis), species (*A. custodiens, C. fulvopilosus* or *T. sericeiventre*) and Group size (1, 5, 10 or 25) as factors and colony of origin as a random factor. To determine the efficacy of grooming behaviours we compared the number of CFUs isolated from dead ants, those maintained alone, treated and untreated individual from a two-member group setting. Comparisons were made with a Kruskal-Wallis test using a Dunns test with Benjamini-Hochberg correction (Benjamini & Hochberg, 1995) for post-hoc testing.

Results

Survival in focal ants did not differ significantly between treatment (control and exposure); however, survival did differ significantly between species with *C. Fulvopilosus* experiencing decreased mortality risks (HR: -1.33, z: -3.985, $p < 0.001$ see Figure 2.4) and *T. sericeiventre* increased mortality (HR: 0.61, z: 2.784, $p < 0.01$ see Figure 2.4) when compared to *A. custodiens*. Furthermore, local density had no effect on survival of the focal ant by itself or as an interaction term with treatment (Figure 2.4).

We found a similar pattern in mortality rates for non-focal individuals with significant differences in species survival. *Camponotus fulvopilosus* depicted a lower mortality risk (HR: -2.00163, z: -10.714, $p < 0.001$ see Figure 2.5) and *T. sericeiventre* a higher hazard ratio (HR: 0.43483, z: 4.869, $P < 0.001$ see Figure 2.5) when compared to *A. custodiens*. At the nestmate level the highest local density decreased mortality risks (HR: -0.92729, z: - 3.081, $p = 0.002$ see Figure 2.5) relative to the lowest local density; however, when compared as an interaction with treatment, did not differ.

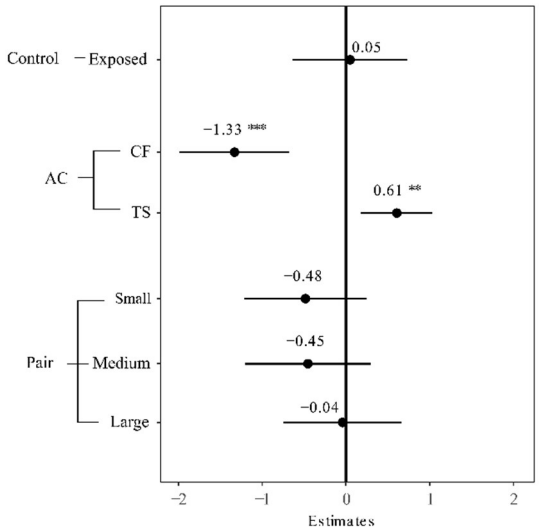

Figure 2.4: Forest plot of the Hazard ratios from a Cox-Proportional hazards model for focal individuals in the local density experiment. The plot depicts the effects of treatment (Control: Tween 20 and Exposed: *Metarhizum anisopliae*, species (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* and TS: *Tetramorium sericeiventre*), group size (Paired, Small: 5 individuals, Medium: 10 individuals or large: 25 individuals) on survival rate. The interaction between group size and treatment on survival rate in focal individuals was not included due to no significance. * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero are non-significant (NS).

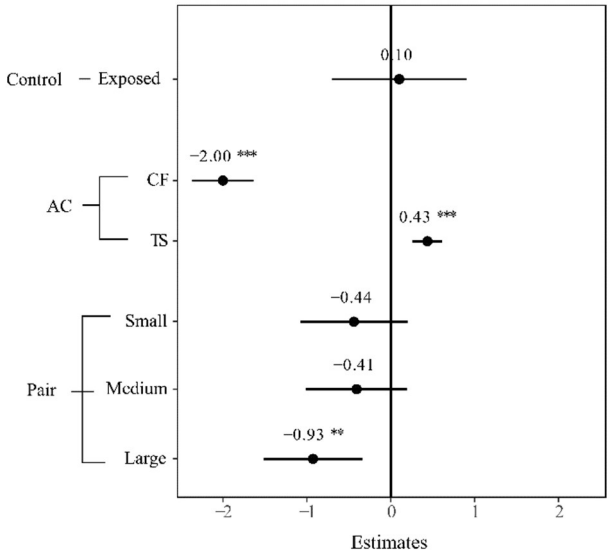

Figure 2.5: Forest plot of the Hazard ratios from a Cox-Proportional hazards model for non-focal individuals in the local density experiment. The plot depicts the effects of treatment (Control: Tween 20 and Exposed: *Metarhizum anisopliae*, species (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* and TS: *Tetramorium sericeiventre*), group size (Pair: one nestmate, small: five nestmates, medium: ten nestmates or large: twenty-five nestmates) on mortality. The interaction between group size and treatment on survival rate in focal individuals was not included due to no significance. * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero are NS.

The focal ant treated with conidia received a small but significant increase in the frequency of interactions compared to ants treated with Tween (β: 0.08762, SE: 0.03805, z: 2.303, p = 0.021 see Figure 2.6 and Table S 2.1). Treated *C. fulvopilosus* ants had significantly fewer interactions when compared to *A. custodiens* (β: - 0.24948, SE: 0.04552, z: - 5.480, p < 0.001 see Figure 2.6 and Table S 2.1) and this was also true for *T. sericeiventre* (β: - 0.28002, SE: 0.04704, z: - 5.952, p < 0.001 see Figure 2.6 and Table S 2.1). Focal ants in exposed and control trials self–groomed much more frequently when compared to the frequency of allogrooming received (β: 1.51853, SE: 0.11352, z: 13.377, p < 0.001 see Figure 2.6 and Table S 2.1). For focal ants, both allogrooming provided (β: - 0.80042, SE: 0.17866, z: - 4.480, p < 0.001 see Figure 2.6 and Table S 2.1) and trophallaxis (β: - 1.16746, SE: 0.20358, z: - 5.735, p < 0.001 see Figure 2.6 and Table S 2.1) were significantly lower than allogrooming received in treated ants.

There were more interactions with the focal control and exposed individual as group size increased, from five (β: 0.43185, SE: 0.13054, z: 3.308, p < 0.001 see Figure 2.6, Table S 2.1 and Figure S 2.1 for marginal effects of interactions), ten (β: 0.57665, SE: 0.12759, z: 4.519, p < 0.001 see Figure 2.6, Table S 2.1 and Figure S 2.1 for marginal effects of interactions) and 25 (β: 0.76881, SE: 0.12358, z: 6.221, p < 0.001 see Figure 2.6, Table S 2.1 and Figure S 2.1 for marginal effects of interactions) when compared to paired ants. Exposed ants provided allogrooming to nestmates less frequently in groups of 5 individuals (β: - 0.77788, SE: 0.26440, z: - 2.942, p = 0.003 see Figure 2.6, Table S 2.1 and Figure S 2.1 for marginal effects of interactions) and 25 individuals (β: - 0.94302, SE: 0.25144, z: - 3.751, p < 0.05 see Figure 2.6, Table S 2.1 and Figure S 2.1 for marginal effects of interactions) when compared to having access to a single nestmate. Self-grooming was also significantly less frequent at local densities of ten (β: - 0.30884, SE: 0.14484, z: - 2.132, p < 0.033 see Figure 2.6, Table S 2.1 and Figure S 2.1 for marginal effects of interactions) and 25 (β: - 0.56639, SE: 0.14179, z: - 3.995, p < 0.05 see Figure 2.6, Table S 2.1 and Figure S 2.1 for marginal effects of interactions) when compared to group sizes of one nestmate. The frequency of interactions with the focal control and exposed ants were less on the second day of observation compared to the first 24 hours when conidia are easier to remove (β: - 0.57984, SE: 0.03959, z: - 14.647, p < 0.001 see Figure 2.6 and Table S 2.1). No significant differences were detected between local density and trophallaxis.

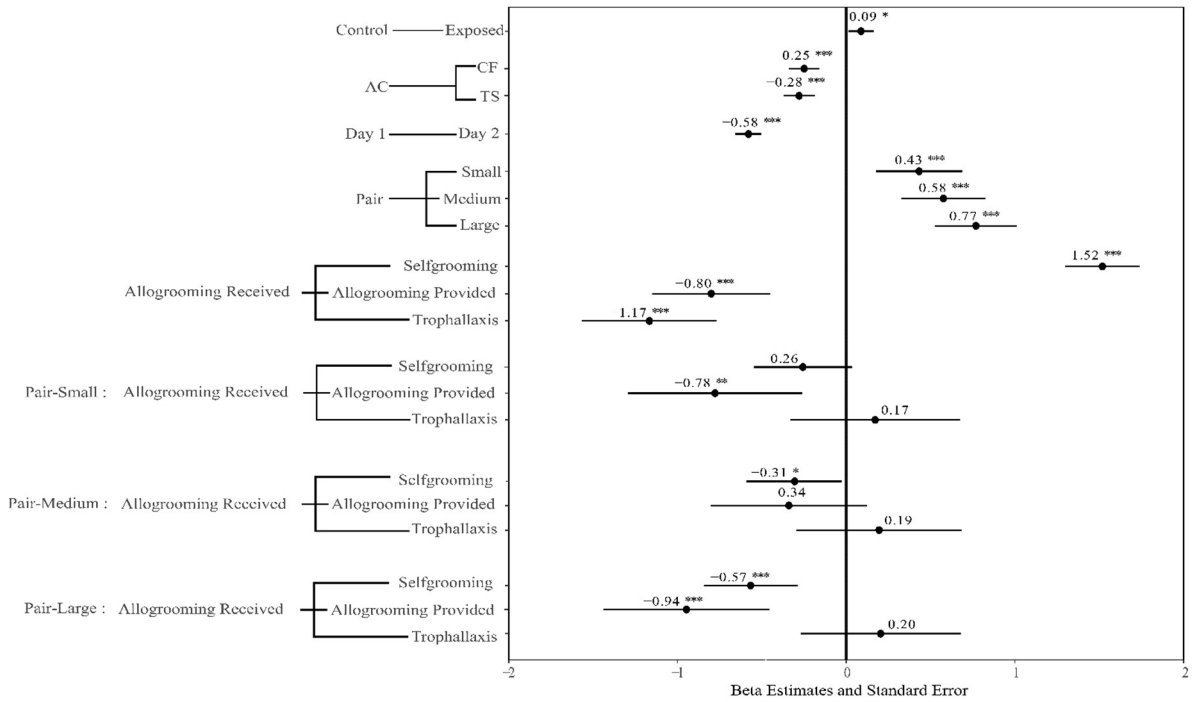

Figure 2.6: Forest plot of Beta estimates and their standard error from a GLMM assessing the effects of treatment (Tween: control or *Metarhizum anisopliae:* exposed*)* on the sanitary behaviour of treated focal ants for different species (AC: *Anoplolepis custodiens,* CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre)* and group size (Pair: one nestmate, small: five nestmates, medium: ten nestmates or large: twenty-five nestmates), day of observation and the interaction between sanitary behaviour and group size on the frequency of sanitary behaviours. * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero are NS.

Exposed focal ants received slight but significantly longer interaction bouts than control focal ants (β: 0.10357, SE: 0.04132, z: 2.506, $p = 0.012$ see Figure 2.7 and Table S 2.2). *Tetramorium sericeiventre* engaged in interactions of shorter duration (β: - 0.33027, SE: 0.05212, z: - 6.337, $p < 0.001$ see Figure 2.7 and Table S 2.2), while *C. fulvopilosus* did not differ in interaction durations when compared to *A. custodiens*. When assessing specific behaviours, we found that focal ants self-groomed for significantly longer periods when compared to interactions with nestmates where they received allogrooming (β: 1.52518, SE: 0.11681, z: 13.057, $p < 0.001$ see Figure 2.7 and Table S 2.2). Focal ants in control and exposed treatments also spent less time donating allogrooming (β: - 0.81305, SE: 0.17925, z: - 4.536, $p < 0.001$ see Figure 2.7 and Table S 2.2) and engaging in trophallaxis (β: - 1.19820, SE: 0.20408, z: - 5.871, $p < 0.001$ see Figure 2.7 and Table S 2.2) than they did receiving allogrooming. We found that as local density increased, focal ants interacted for longer, within groups of five nestmates (β: 0.50564, SE: 0.13195, z: 3.832, $p < 0.001$ see Figure 2.7 and Table S 2.2), ten (β: 0.69730, SE: 0.12906, z: 5.403, $p < 0.001$ see Figure 2.7 and Table S 2.2) and 25 (β: 0.90123, SE: 0.12559, z: 7.176,

$p < 0.001$ see Figure 2.7 and Table S 2.2) engaging in significantly longer interaction events compared to focal ants with a single nestmate.

When assessing the interaction of how local density affected behaviours we found that focal ants in small groups (5 nestmates) when compared to pairs, provided shorter bouts of allogrooming (β: - 0.87222, SE: 0.26530, z: - 3.288, $p = 0.001$ see Figure 2.7, Table S 2.2 and Figure S 2.2 for marginal effects of interactions) and self-grooming (β: - 0.31914, SE: 0.15342, z: - 2.080, $p = 0.038$ see Figure 2.7, Table S 2.2 and Figure S 2.2 for marginal effects of interactions) than receiving allogrooming. A similar pattern was detected in large groups (25 nestmates) with focal ants engaging in shorter bouts than pairs for both self-grooming (β: - 0.76027, SE: 0.14817, z: - 5.131, $p < 0.001$ see Figure 2.7, Table S 2.2 and Figure S 2.2 for marginal effects of interactions) and donating allogrooming 25 (β: - 1.08387, SE: 0.25261, z: - 4.291, $p < 0.001$ see Figure 2.7, Table S 2.2 and Figure S 2.2 for marginal effects of interactions) when compared to bouts of receiving grooming. Medium sized groups, when compared to ant pairs only, self-groomed for less time than received allogrooming interactions (β: - 0.41408, SE: 0.15038, z: - 2.754, $p = 0.006$ see Figure 2.7, Table S 2.2 and Figure S 2.2 for marginal effects of interactions). Finally, focal ants spent much less time interacting on the second day post exposure compared to the first day (β: - 0.68091, SE: 0.04266, z: - 15.963, $p < 0.001$ see Figure 2.7 and Table S 2.2).

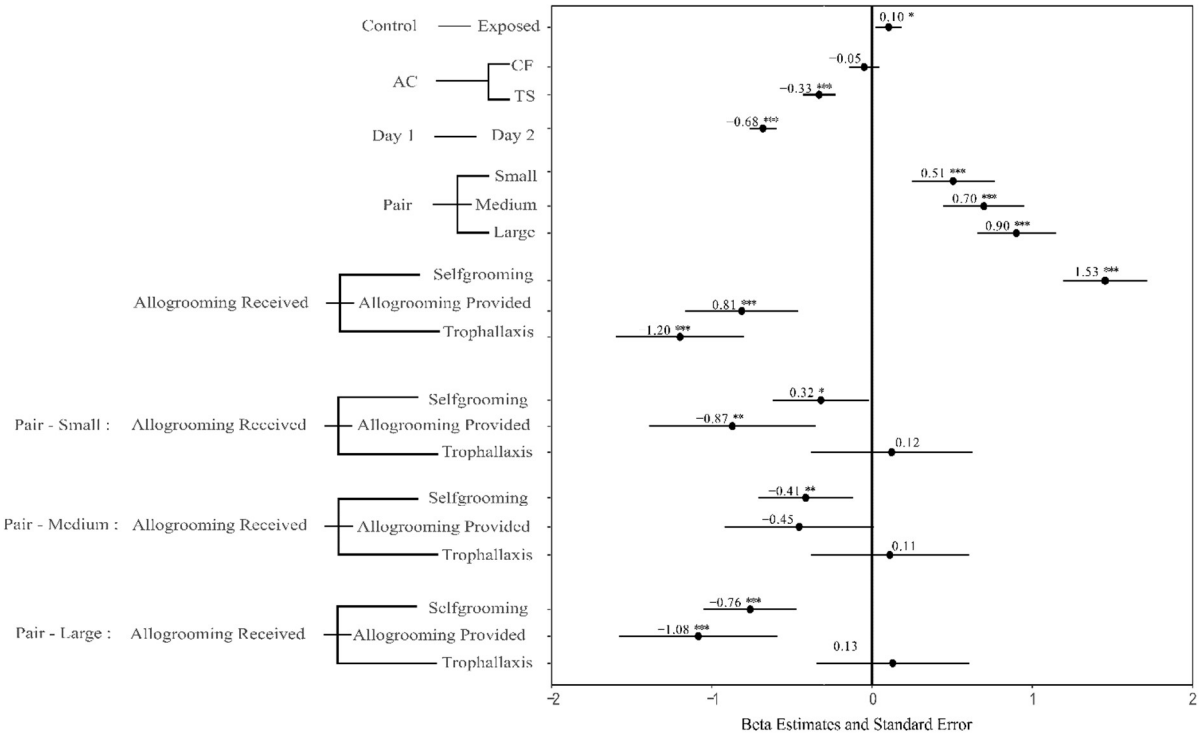

Figure 2.7: Forest plot of Beta estimates and their standard error from a GLMM assessing the effects of treatment (Tween: control or *Metarhizum anisopliae:* exposed*)*, species (AC: *Anoplolepis custodiens,* CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre)*, sanitary behaviour, group size (Pair: one nestmate, small: five nestmates, medium: ten nestmates or large: twenty-five nestmates), day of observation and the interaction between sanitary behaviour and group size on the duration of sanitary behaviours. * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero are NS.

We found that the efficacy of conidia control was influenced by whether an exposed individual was isolated or paired with a nestmate. In *A. custodiens,* the number of conidia remaining after 24-hours of grooming and/or isolation differed significantly across treatments (Kruskal–Wallis: $H_3 = 348.43$, $p < 0.001$, Figure 2.8 A). Cadavers had the greatest conidia load, while untreated nestmates had the lowest, further access to allogrooming resulted in fewer conidia when compared to ants which only had access to self-grooming. Similarly, *T. sericeiventre* showed significant differences in number of conidia removed between treatments (Kruskal–Wallis: $H_3 = 431.53$, $p < 0.001$, Figure 2.8 C), showing the same pattern as *A. custodiens.* Overall, *C. fulvopilosus* treatments differed (Kruskal–Wallis: H_3,=401.08, $p < 0.001$, Figure 2.8 B); however, isolated ants did not differ from ants maintained with a nestmate, with self- and allogrooming lowering conidia loads but not differing significantly from each other. Additionally, secondary transfer to untreated nestmates was negligible.

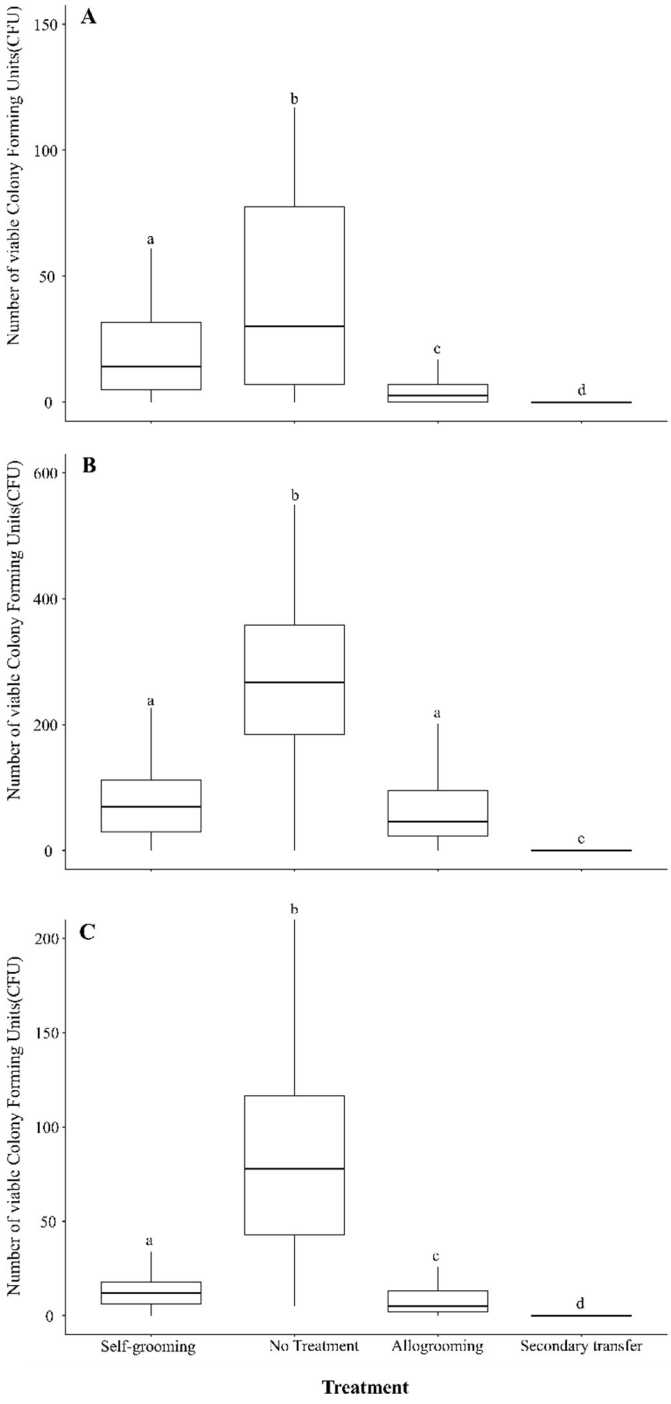

Figure 2.8: Number of Colony Forming Units (CFU) of *Metarhizum anisopliae* grown from the conidia remaining on an exposed ant after 24 hours with access to different sanitary regimes in A: *Anoplolepis custodiens*, B: *Campanotus fulvipilosus* and *C: Tetramorium sericeiventre*. Exposed ants were killed prior to exposure to assess conidia counts in the absence of sanitary behaviour (No treatment). Ants were maintained alone after exposure to assess the efficacy of selfgrooming (Self-grooming). Finally ants were maintained with a nestmate to assess the efficacy of allogrooming in conjuction with self grooming (Allogrooming) and to assess the risk of conidia transfer to an untreated nestmate during allogrooming (Secondary transfer). Treatments that are significantly different are followed by different letters, $p < 0.01$, Dunn's post hoc test. Solid lines depict medians, boxes represent interquartile range, whiskers represent min and max.

Discussion:

The sanitary behaviours tested here occur more frequently and for longer durations with increased group size, suggesting a potential relationship between the rate of sanitary grooming and local density of nestmates; however, this did not hold for trophallaxis. Pathogen-exposed individuals frequently self-groomed and for longer periods than the allogrooming received from nestmates. Yet they did receive more allogrooming from nestmates and these exchanges lasted longer than the allogrooming donated to non-exposed nestmates. Interestingly, we found that pathogen exposed, and control exposed individuals had similar mortality rates, and even though the three species varied in their rates of mortality, the conidia removed during allogrooming and self-grooming were substantial, rendering these behaviours as effectual prophylactic strategies.

We found that mortality rates did not differ between pathogen exposed and control exposed ants collectively for all species, which suggests that sanitary behaviours are extremely effective at mitigating exposure risks during this early phase. Previous studies frequently find that mortality is higher in fungal infected treatments when compared to controls (Hughes & Boomsma, 2004; Reber *et al.*, 2011; Ho & Frederickson, 2014; Leclerc & Detrain, 2018). A potential explanation for our finding is that the treatment used in this study was insufficient to cause infection; however, the treatment (conidia density) is in line with other studies (Storey *et al.*, 1991; Vergeer *et al.*, 2003; Hughes *et al.*, 2004; Reber *et al.*, 2011; Schmidt *et al.*, 2011; Purcell *et al.*, 2012; Brütsch & Chapuisat, 2014; Ho & Frederickson, 2014; Tranter *et al.*, 2015; Qiu *et al.*, 2016) and further when piloted the treatment led to mortality as a result of infection in all species. A more reasonable explanation for the lack of differences in mortality across treatments is that the pathogen loads in treated individuals were mitigated through sanitary behaviours allowing them to overcome the pathogen challenge.

The effectiveness of sanitary behaviour as an early phase mechanism mitigating exposure was evident with the frequency and duration of sanitary behaviours towards an exposed individual decreasing after 24 hours. This suggests that the upregulation of sanitary interaction is context specific with a greater care given initially when interventions have the most impact. This is particularly important as entomopathogens germinate within 24-48 hours, and *M. anisopliae* can germinate and form attachment structures as early as 12-24 hours (Hajek & Leger, 1994; Moino *et al.*, 2002) after which conidia cannot be removed from the cuticle of the infected host.

Thus, the upregulation of interaction during this window of opportunity is extremely important in limiting infections.

Furthermore, this pattern of decreasing interaction over time could suggest active pathogen monitoring by ants. This is supported by the fact that ants have been shown to treat fungus-killed cadavers more intensely than cold-killed cadavers (Diez *et al.*, 2015) and to upregulate grooming in response to infection (Theis *et al.*, 2015) in a dose dependent manner (Reber *et al.*, 2011). Further support for this argument could be expanded through experimental work assessing plasticity in grooming in response to sequential introductions of varying intensity of fungal challenged foragers. Alternatively, this may represent a general behavioural syndrome wherein workers returning to the group are prioritised regardless of infection status, as has been detected in *Atta* (Morelos-Juárez *et al.*, 2010) and consequently nestmate exchanges will decrease over time.

This increased rate and time spent on sanitary behaviour toward exposed individuals is potentially adaptive. We found that both the frequency and duration of interactions increased in response to pathogen exposure, albeit weakly, suggesting that ants can upregulate interaction rates (Hughes & Boomsma, 2004; Walker & Hughes, 2009). Exposed individuals primarily engaged in self-grooming, which represents a first line of defence against a potential pathogen (Morelos-Juárez *et al.*, 2010; Theis *et al.*, 2015). However, the investment in self-grooming decreased as group size increased suggesting that access to more nestmates ameliorated the burden on exposed individuals as has been previously detected (Traniello *et al.*, 2002; Reber *et al.*, 2011; Okuno *et al.*, 2012). Similar to other studies, we found that exposed individuals were also more likely to receive allogrooming then donate it (Reber *et al.*, 2011; Theis *et al.*, 2015), likely due to the risk they present to their nestmates. This could also be argued as a form of self-exclusion (Bos *et al.*, 2012), but further experimentation would be required to confirm this. Trophallaxis occurred infrequently and interaction rates did not change with group size, suggesting it played little to no role in managing pathogen exposure and continued to function primarily in resource transfer.

Given our findings on the reliance of high levels of self-grooming and allogrooming we determined the efficacy of these behaviours in reducing conidia load. Both sanitary behaviours, namely self- and allogrooming, were effective in reducing conidia loads, confirming these behaviours as primary mechanisms for pathogen control. Cadavers always had the highest

conidia load across all species, reinforcing the effectiveness of sanitary behaviours. The risk of an exposed individual transferring conidia to a nestmate during allogrooming was extremely low, as has been found previously (Reber *et al.*, 2011; Konrad *et al.*, 2012). This suggests that the costs incurred through allogrooming are extremely low and may even be beneficial through the action of immune priming (Traniello *et al.*, 2002; Masri & Cremer, 2014; Liu *et al.*, 2015). Given the extremely low level of conidia transfer, groomers are unlikely to succumb to the fungal pathogen and can instead prepare their immune system for subsequent challenges. Yet, the potential benefits accrued from immune priming may be offset by costs incurred as a result of infections from other pathogens. A recent study showed that individuals primed to one EPF species, whilst experiencing greater survival with a subsequent infection of the homologous EPF, showed higher mortality rates when infected with a heterologous EPF (Konrad *et al.*, 2018). This suggests that ants may need to modify their investment in grooming based on the likelihood of multiple pathogen challenges and their micro habitat.

Whilst self-grooming may be the primary mechanism for pathogen control, access to a nestmate and allogrooming led to a further decrease in conidia load in *A. custodiens* and *T. sericeiventre,* suggesting that allogrooming promotes increased conidia control and possibly limits the likelihood of succumbing to an EPF challenge (Okuno *et al.*, 2012). Even though *A. custodiens* engaged in the most interactions and for the longest periods, they did not have the lowest mortality rates. This could suggest that their sanitary behaviours are not as effective as other species. A more parsimonious explanation is that this represents a dominant species which is abundant in the study area, establishing large, polydomous nests. Thus, this species might better tolerate (Scharf *et al.*, 2012; Pull *et al.*, 2013) small epidemics compared to less prolific species and therefore need not invest as heavily in their sanitary behaviour. Yet they still engage in sanitary behaviours more often and for longer than other species, which suggests greater investment to offset the lack of efficacy. Interestingly, *T. sericeiventre* had the lowest survival and were least reliant on sanitary interactions with treated individuals. In pilot studies when individuals where exposed to the pathogen, in the absence of nestmates, survival was greatest in *T. sericeiventre,* suggesting that they may be less reliant on SI mechanisms due to increased individual immunity. Yet in this study, in the presence of nestmates, survival was lowest in *T. sericeiventre*, revealing the important role of SI in overcoming the limitations of innate immunity. Given their habitat is more mesic in nature, it is likely that they have had to

evolve alternate defences against fungal pathogens, as has been shown in leaf cutting ants that elect to place their garbage in dryer nest chambers (Ribeiro & Navas, 2007).

Camponotus fulvopilosus relied less on allogrooming, with pathogen load minimised equally when ants were isolated or had access to a nestmate. This implies that allogrooming offers no added benefit to conidia control in this species. But it is unlikely that *C. fulvopilosus* are not making use of alternate mechanisms for conidia control since they had the lowest mortality rates in this study. Despite having the highest survival rates, *C. fulvopilosus* also had the highest conidia load after interventions and did not increase conidia management when provided access to allogrooming. This suggests that they may employ alternate mechanisms in resisting fungal pathogens. One explanation is their thick cuticle, which reduces water loss in their xeric environment during long solitary foraging bouts, may help limit susceptibility to EPFs (David, 1967). An alternate explanation could be their liberal use of formic acid. In the Formicidae, formic acid alone accounts for up to 70% of the inhibitory effect of venom, furthermore whilst acetic acid has no effect on its own, it works synergistically with formic acid to increase the anti-microbial effects of venom (Tragust et al. 2013). Personal observations of *C. fulvopilosus* show that they frequently react to threats by spraying venom from their acidopores. We observed a fascinating behaviour in *C. fulvopilosus*, most likely a form of collective disinfection, whereby individuals spontaneously aggregate and groom their acidopores, releasing a potent formic acid mixture (Figure S 3.1). This behaviour was also detected in *A. custodiens* (Figure S 3.2). Further research is required to assess this behaviour and determine its pathogen control capacity.

An important aspect in social insect societies is local density, as high numbers of closely related individuals are expected to be at risk from pathogen incursions (Schmid-Hempel, 1998). When assessing the frequency and duration of sanitary behaviours we found that increasing the number of nestmates within a treated individuals environment resulted in longer and more frequent interactions. This fits with our expectations of DDP (Wilson & Reeson, 1998) and with findings that increasing colony size has been linked to increased task specialisation (Holbrook *et al.*, 2011). Higher local densities may lead to more intense grooming, as has been detected on small scales (Okuno *et al.*, 2012), to offset the increased risk accrued as a result of proximity within complex interaction networks. The importance of increased grooming in response to increased group size needs further exploration, as one study assessing group size effects found no difference in conidia removal between ants maintained in small or large groups

(Reber *et al.*, 2011); however, this was assessed over a very short time frame. As such, given the plasticity in grooming related to group size found in this and other studies, it suggests that further research should assess how group size affects conidia removal in a time dependent manner during the window when conidia attachment is low (Vestergaard *et al.*, 1999).

We set out to assess how South African ants alter sanitary interactions in order to control challenge by a fungal entomopathogen. We assessed changes during the period when conidia adherence is lowest and sanitary behaviours may have the greatest effect. We found that South African ants were able to upregulate sanitary behaviours in response to infection, were able to effectively make use of this window of opportunity, particularly in the first 24 hours, and remove sufficient conidia to minimise infection risk and subsequent mortality. Further we discovered that investment in these behaviours were density dependent with larger groups increasing their allogrooming of infected individuals and self-grooming decreasing in larger groups. We found species specific responses with *A. custodiens* engaging in more sanitary behaviours than *C. fulvopilosus* and *T. sericeiventre.* Future studies should assess the importance of time dependent conidia removal rates, assess *A. custodiens* and *C. fulvopilosus'* potential group level disinfection and explore shifts in sanitary behaviours at the colony level. Finally, this study highlights the importance of assessing multiple species while including under-represented geographical regions.

Research contributions

DJP, with aid from TCW, conceived and designed the experimental setup and analysis. DJP implemented the experimental setup, collected and analysed the data, with aid from Welri Nortje in watching and annotating observations. DJP wrote the paper with input from TCW and CH.

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Chapter 3: Prophylactic and activated colony-level responses to a pathogen challenge in three South African ant genera.

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Abstract:

Colonies of social insects, which include ants, benefit from group living through access to division of labour. Yet these benefits are offset by increased pathogen risk. Ants use social immunity to mitigate these costs prophylactically and respond actively to pathogen exposure. Ants protect the queen and brood by participating in allogrooming to remove pathogenic particles and trophallaxis for the transfer of information or antimicrobials. These behaviours are expected to differ across ant species based on their life history, ecology and pathogen pressure. We assessed the frequency and duration of allogrooming and trophallaxis in experimental colonies of three species of South African ants, *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre* under standard laboratory conditions, Tween control conditions or in response to pathogen exposure to *Metarhizium anisopliae.* The frequency of allogrooming increased and the frequency of trophallaxis decreased most in pathogen exposed colonies, followed by Tween treated colonies, this was driven primarily by changes in *A. custodiens,* followed by changes in *T. sericeiventre* behaviour. The duration of allogrooming increased in treated *C. fulvopilosus* but decreased for pathogen exposed, *T. sericeiventre. Camponotus fulvopilosus* and *T. sericeiventre* upregulated trophallaxis duration when treated. All three species engaged in allogrooming and trophallaxis, and altered their frequency and duration in response to pathogen exposure, mitigating pathogen risks. Species differed in their responses with *A. custodiens* engaging and adjusting their response most when encountering pathogen exposed nest mates.

Intended for submission to "Journal of insect Science", "Behavioural Processes" or "Insects Sociaux"

Introduction

The division of reproductive labour facilitates the ecological success of complex societies, such as those of social insects. Owing to their success these colonies can reach staggeringly large sizes (Wilson, 1971). Dense colony populations coupled with extensive genetic relatedness are expected to lead to increased risk from infectious agents (Hölldobler & Wilson, 1990). This proposed propensity to infections is further exacerbated by frequent interactions (Read *et al.*, 2008; Bansal *et al.*, 2010), in the form of resource sharing via trophallaxis, information transfer via antennation and frequent grooming of nestmates (Hölldobler & Wilson, 1990). In order to combat these risks individuals within a colony implement behaviours to decrease pathogen risks, potentially at a cost to themselves, and have collectively been termed social immunity (SI; Cremer *et al.*, 2007). These behaviours can be prophylactic in nature or activated in response to infection.

Prophylactic behaviours in general, focus on modifications to interaction networks (Hart & Ratnieks, 2001; Naug & Camazine, 2002; Griffiths & Hughes, 2010; Mersch *et al.*, 2013; Stroeymeyt *et al.*, 2014), removal of potential infectants (Ortius-Lechner *et al.*, 2000; Hughes *et al.*, 2002; Diez *et al.*, 2014) as well as the implementation of antiseptics (Chapuisat *et al.*, 2007; Simone-Finstrom & Spivak, 2012; Brütsch & Chapuisat, 2014). However, of interest here are activated responses, where colonies adapt to pathogen intrusion by modifying behaviours, for example altering allogrooming and trophallaxis rates. Allogrooming benefits the colony by removing or inactivating pathogen loads on nestmates' cuticles (Loreto *et al.*, 2014). Rates of allogrooming have been shown to increase in response to challenges of surface borne pathogens (Hughes *et al.*, 2002; Walker & Hughes, 2009; Bos *et al.*, 2012; Okuno *et al.*, 2012), with nestmates engaging more frequently in allogrooming to decrease pathogen loads of infected colony members. A comparative study between leaf-cutting, harvester, wood and weaver ants showed that all species were able to detect pathogens and modify their behaviour; however, the species responded differently, with one increasing their self-grooming rates whilst another relied more on allogrooming (Tranter et al., 2014b). Social insects engage primarily in trophallaxis to share resources between colony members (Hölldobler & Wilson, 1990); yet, it has been proposed that it may also facilitate the sharing of immune system elicitors and antimicrobials (Hamilton *et al.*, 2011). Given the potential importance of these two mechanisms of activated SI, it questions how ubiquitous an investment these behavioural modifications are across species.

It is clear from previous studies that not all ant species invest equally in immune behaviours or adopt the same mechanisms when challenged with disease. For example, arborealism is associated with lower levels of soil-borne fungi and consequently Walker & Hughes (2011) predicted that, in general, arboreal species would encounter fewer diseases and thus invest less in disease defence behaviours. They found terrestrial and arboreal species differed significantly across seven neotropical species of ants, yet some terrestrial and arboreal species invested similarly in disease defence behaviours and concluded that immune investment was associated with high diversity of pathogens (Walker & Hughes, 2011) and not life history. Metapleural glands are unique to ants and their secretions are antimicrobial (see Yek & Mueller, 2011 for review) but not all species have these glands and a comparison between weaver ants without metapleural glands revealed that one species relied on venom gland secretions whilst the other relied on increased grooming rates (Tranter & Hughes, 2015). Degree of melanisation may also lead to variation in natural immunity as it facilitates immune defences (Wilson *et al.*, 2001; Sinotte *et al.*, 2018). Past exposure to pathogens may facilitate immune priming (Konrad *et al.*, 2012; Masri & Cremer, 2014), hence colonies inhabiting fungi-rich soils, such as mesic environments, may have a greater innate immunity. Colony size is also expected to play a role in disease defence, with larger colonies found to remove infectious particles from the nest quicker than smaller colonies (Leclerc & Detrain, 2018). Further, smaller colonies may simply abandon their nests to facilitate sanitization (Drees *et al.*, 1992; Leclerc & Detrain, 2018).

Assessments of species responses to pathogen challenges have shown strong geographic bias, with no sub-Saharan species assessed to date. Furthermore, most studies frequently assess SI without including brood and a reproductive, for example a queen. While this is important for quantifying behavioural responses on individual and group levels it does not account for colony level responses which are expected to play key roles in epidemic mitigation (Cremer *et al.*, 2007; Stroeymeyt *et al.*, 2014). In particular, the inclusion of a queen and brood could facilitate division of labour by increasing the breadth of tasks to be managed, whilst also introducing consequences from failures in pathogen control. Studies assessing SI in the absence of a queen may overlook nuanced behavioural regulation resulting from the costs associated with infection in queens. A study assessing colony responses towards infected brood showed that nest-workers will euthanise and destructively disinfect infected brood (Pull *et al.*, 2018). In a network analysis of more than nine million interactions, researchers identified three major behavioural groups (nurses with the queen, cleaners and foragers) in colonies of *Camponotus fellah* and found individuals interacted more within their group than with individuals from

another group (Mersch *et al.*, 2013). This compartmentalised interaction structure is suggested to help mitigate epidemics or pathogen exposure towards important individuals by localising infections within compartments (Cremer *et al.*, 2018). Furthermore, laboratory studies which, include brood and a queen more closely resemble field conditions by virtue of increased threat through the loss of the colony's reproductive.

Here we assess how the frequency and duration of both allogrooming and trophallaxis shift in response to infection across three South African ant species *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre*. As previously mentioned, *A. custodiens* is an ecologically dominant native ant species (Samways, 1990; Addison & Samways, 2000) which occurs in semi-arid environments, while *C. fulvopilosus* represents an arid-adapted species with a wide distribution and is known to spray formic acid up to 15 cm through the air to defend the nest (Robertson & Zachariades, 1997). Finally, *T. sericeiventre* represents an opportunist ant species that has been shown to coexist with many species, including invasive species such as Argentine ants (Hoffmann & Andersen, 2003; Luruli, 2007). *Tetramorium sericeiventre* has a wide distribution across Africa and across environmental clines, likely resulting in an evolutionary history characterised by diverse pathogen interactions. These three species inhabit a wide range of environments but also encompass diverse life histories, such as solitary and coordinated foraging, polydomous and monodomous colonies as well as competitive exclusion and opportunistic foraging.

Behavioural interactions of workers, namely allogrooming and trophallaxis, were assessed upon the return of pathogen-exposed foragers to queenright colonies containing brood. We expect that numerically large *A. custodiens* colonies will upregulate interaction frequencies and durations in response to infection, as large colonies are able to deal with infections more swiftly than small colonies (Leclerc & Detrain, 2018). We predict that *C. fulvopilosus* will not show significant shifts in interaction frequencies and durations due to their potential reliance on chemical control through formic acid (Tranter & Hughes, 2015) and their naturally melanised cuticles, which have been shown to increase innate immunity (Wilson *et al.*, 2001). Finally shifts in interaction rates in T. *sericeiventre* are expected to be negligible, assuming they have a higher innate immunity, which results from encountering more fungal pathogens in mesic soils and through their coexistence with multiple ant species (Konrad *et al.*, 2012; Masri & Cremer, 2014).

Methods

Experimental setup

Three colonies each of *A. custodiens*, *T. sericeiventre* and *C. fulvopilosus* were collected and maintained as described in Chapter two. Again, the generalist insect pathogen, *Metarhizium anisopliae* (META69, isolate: ICIPE69) was used in all experiments since it is frequently employed in SI studies (Traniello *et al.*, 2002; Hughes & Boomsma, 2004a; Pie *et al.*, 2005; Reber & Chapuisat, 2012; Tragust *et al.*, 2013b; Tranter *et al.*, 2015; Qiu *et al.*, 2016). The fungus was cultivated, and the conidia solution prepared as described in Chapter two.

Experimental nests were created by using two panels of clear antireflective glass (15 X 15 X 1 cm) separated with a nest chamber cut-out made from JoinTech (see Figure 3.1). Nest designs differed per species with total available space proportional to average nest worker size, *A. custodiens* (±800 mm^2 X 5 mm height, Figure 3.1 A), *C. fulvopilosus* (±1300 mm^2 X 10 mm height, Figure 3.1 B) and *T. sericeiventre* (±500 mm^2 X 3 mm height, Figure 3.1 C). Experimental nests were attached to foraging arenas (32 X 21.5 X 7.5 cm) via plastic tubing. Nests were placed within filming boxes (23 X 20.5 X 51 cm) to minimise light. Each camera box contained a HDCVI camera (Dahua) with modified IR filters and an infrared light source. Ants are unable to detect infrared light and thus the colonies experienced dark conditions simulating natural nest conditions. Within the foraging arena, water and a 20% sugar solution were provided ad libitum along with a protein source every three days, in the form of termites. Arenas were maintained in a temperature (± 25 ºC) and humidity-controlled environment with a 12-hour day night cycle (Figure 3.2).

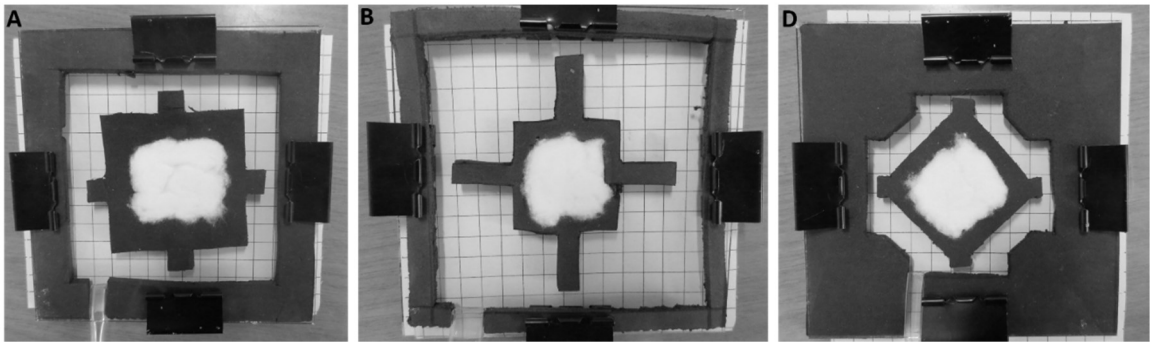

Figure 3.1: Experimental nest structures, consisting of two plates of non-reflective glass separated by JoinTech to create nest structuring. The central section contained cotton wool soaked in H2O. Nest structures were created per species to create a nest space proportional to average worker size. Three nest designs were created. A: for *Anoplolepis custodiens (±800 mm^2)* B: for *Camponotus fulvopilosus (±1300 mm^2)* and C: for *Tetramorium sericeiventre (±500 mm^2)*.

Figure 3.2: Experimental nest (A), consisting of two sheets of non-reflective glass separated by JoinTech cut to form nest structures. The experimental nest was housed in a light excluding recording box lit with a camera and infrared light source (B). The experimental nest was attached using plastic tubing (C) to a foraging arena (D) lined with Fluon where sugar water was provided ad libitum along with termites every three days as a protein source.

Three experimental colonies per species were set up with 100 worker ants, a queen and a mixture of brood; eggs, larvae and pupae (AC: ±1.5 cm^2, CF: ±3 cm^2 and TS: ±1 cm^2). Experimental nests were allowed a 24-hour acclimation phase, before any ants found in the foraging arena were marked (POSCA PC-5 marker) on their abdomen, signifying their status as foragers. Forager checks were repeated three times daily for the duration of the trial to identify and mark any new foragers in the arena. Colonies were then recorded for 48 hours representing baseline conditions for comparison against a control and pathogen exposure treatment. For the control treatment, 10 foragers from each colony were sprayed, as per previous protocol (Chapter 1), with a 5% Tween 20 solution, marked on their thorax and reintroduced to the arena. Colonies were maintained for a further 48 hours before the process was repeated with an additional 10 foragers being sprayed, as per previous protocol (Chapter 1), with a fungal solution of 1 x 10^7 conidia per ml 5% Tween 20 solution, marked on their head and returned to the foraging arena representing the "Pathogen exposed" treatment. Colonies were maintained for a final 48 hours. Ten-minute video recordings were collected

every four hours during the 48-hour treatment periods (Baseline, Control and Exposed), resulting in twelve recordings per treatment and colony to be used for subsequent analyses.

During each 10-minute observation, we identified any allogrooming or trophallaxis events and assessed the duration of the event as well as the total number of allogrooming or trophallaxis events per 10-minute period. Only contacts lasting longer than three seconds were recorded. Trophallaxis was characterised by mouth to mouth contact whilst allogrooming was characterised by mouth to body contact, with directionality assessed in allogrooming.

Statistical Analysis

All analyses were implemented in the R statistical environment using base R (R-Core-Team, 2013) and the package "GlmmTMB" (Nielsen *et al.*, 2017). In order to assess changes in the frequency and duration of sanitary behaviours we implemented generalised linear mixed models (GLMM). We applied a Poisson family to the frequency and duration of allogrooming and trophallaxis, with treatment as the fixed factors (baseline, control and infection), species (*A. custodiens, C. fulvopilosus* and *T. sericeiventre*) and their interaction together with colony as a random effect.

Results:

Workers allogroomed more frequently in response to both the Tween treatment (β: 0.261930, SE: 0.117879, z: 5.86, $p < 0.001$; see Figure 3.3 and Table S 3.1) and *Metarhizium anisopliae* challenge (β: 0.447145, SE: 0.043041, z: 10.39, $p < 0.001$; see Figure 3.3 and Table S 3.1). Colonies of *A. custodiens* allogroomed significantly more than *C. fulvopilosus* (β: -2.284557, SE: 0.194711, z: -11.73, $p < 0.001$ see Figure 3.3 and Table S 3.1) or *T. sericeiventre* (β: -0.424563, SE: 0.168501, z: -2.52, $p = 0.012$ see Figure 3.3 and Table S 3.1). Context affected allogrooming frequency, with the interaction of species and treatment evident for *C. fulvopilosus* which increased allogrooming compared to *A. custodiens* in response to Tween (β: 0.393629, SE: 0.139372, z: 2.82, $p = 0.005$ see Figure 3.3, Table S 3.1 and Figure 3.7 A for marginal effects of interactions). Conversely, T. *sericeiventre* decreased their allogrooming frequency in comparison to *A. custodiens* in response to pathogen exposure (β: -0.425092, SE: 0.072427, z: -5.87, $p < 0.001$ see Figure 3.1, Table S 3.3 and Figure 3.7 A for marginal effects of interactions).

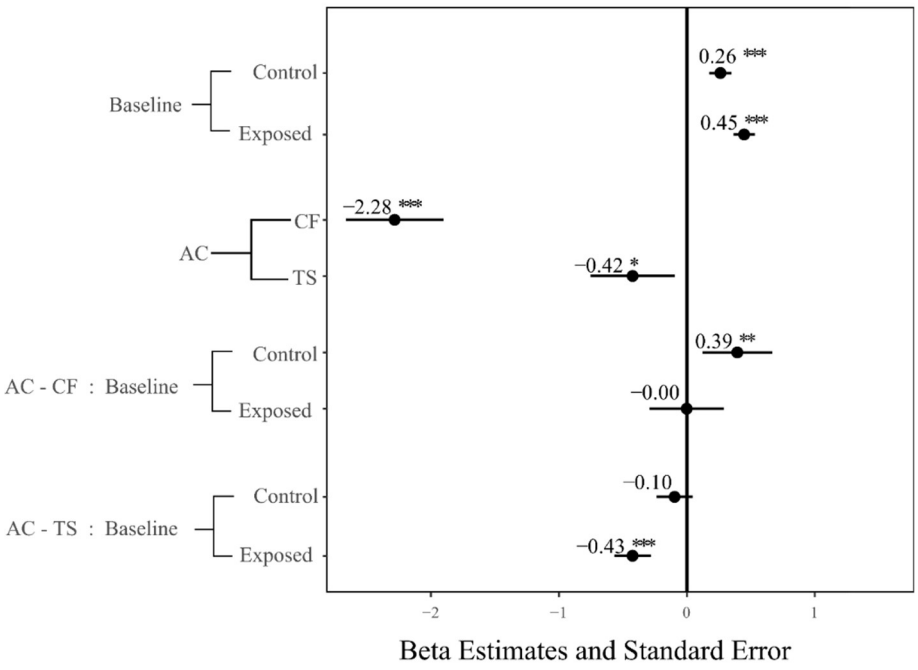

Figure 3.3: Forest plot of Beta estimates and standard error from a GLMM assessing the effects of species (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre*) and treatment (Baseline, Tween control, Exposed to *Metarhizium anisopliae*) as well as the interaction between species and treatment on the frequency of allogrooming. * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero are NS.

In response to pathogen exposure, workers allogroomed for slightly shorter periods (β: -0.0183113, SE: 0.0060048, z: -3.05, p = 0.002 see Figure 3.4 and Table S 3.2); however, no change in allogrooming times were detected for Tween. *Tetramorium sericeiventre* allogroomed for significantly longer than *A. custodiens* (β: -0.0183113, SE: 0.0060048, z: -3.05, $p < 0.001$ see Figure 3.4 and Table S 3.2). *Anoplolepis custodiens* and *C. fulvopilosus* allogroomed for similar times; yet, this was particularly context dependent with the interaction of species and treatment playing a large role. *Camponotus fulvopilosus* responded to both Tween (β: 0.4971951, SE: 0.0179444, z: 27.71, $p < 0.001$ see Figure 3.4, Table S 3.2 and Figure 3.7 C for marginal effects of interactions) and the fungal challenge (β: 0.4815306, SE: 0.0188372, z: 25.56, $p < 0.001$ see Figure 3.4, Table S 3.2 and Figure 3.7 C for marginal effects of interactions) by increasing the duration of their allogrooming in comparison to *A. custodiens*. In contrast, *T. sericeiventre* decreased their duration of allogrooming when exposed to Tween (β: -0.425092, SE: 0.072427, z: -5.87, $p < 0.001$ see Figure 3.4, Table S 3.2 and Figure 3.7 C for marginal effects of interactions) and a fungal challenge (β: -0.425092, SE: 0.072427, z: -5.87, $p < 0.001$ see Figure 3.4, Table S 3.2 and Figure 3.7 C for marginal effects of interactions) compared to *A. custodiens*.

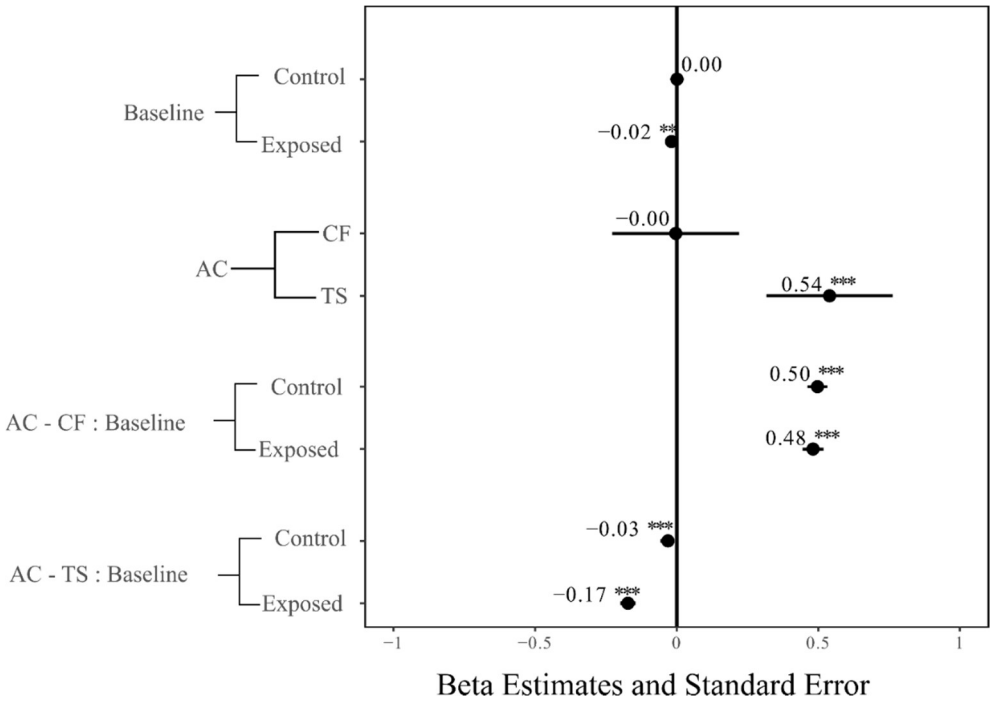

Figure 3.4: Forest plot of Beta estimates and standard error from a GLMM assessing the effects of species (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre*) and treatment (Baseline, Tween control, Exposed to *Metarhizium anisopliae*) as well as the interaction between species and treatment on the duration of allogrooming. * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero are NS.

We found that ants responded to pathogen exposure overall by engaging in less trophallaxis (β: -0.26258, SE: 0.06981, z: -3.76, $p < 0.001$ see Figure 3.5 and Table S 3.3); however, *A. custodiens* engaged in more trophallaxis than both *C. fulvopilosus* (β: -0.69355, SE: 0.14901, z: -4.65, $p < 0.001$ see Figure 3.5 and Table S 3.3) and *T. sericeiventre* (β: -1.22133, SE: 0.15865, z: -7.70, $p < 0.001$ see Figure 3.5 and Table S 3.3). Context did affect trophallactic frequency in that the interaction between species and pathogen exposure was no longer significant. *T. sericeiventre* engaged in more trophallactic interactions compared to *A. custodiens* when exposed to Tween (β: 0.31545, SE: 0.13183, z: 2.39, $p = 0.017$ see Figure 3.5, Table S 3.3 and Figure 3.7 B for marginal effects of interactions)

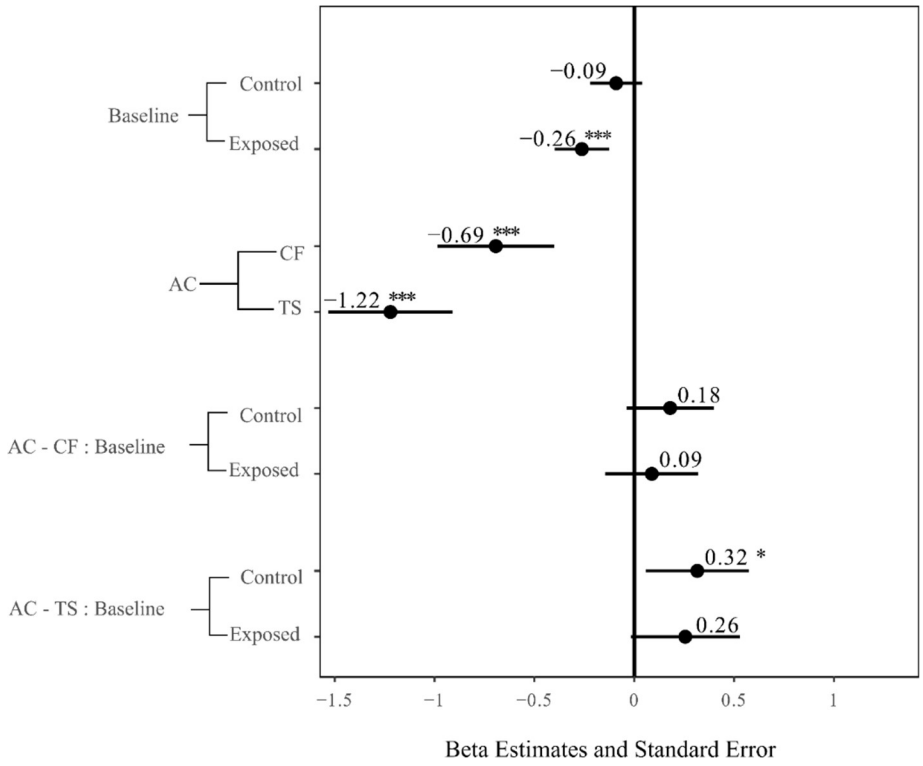

Figure 3.5: Forest plot of Beta estimates and standard error from a GLMM assessing the effects of species (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre* and treatment (Baseline, Tween control, Exposed to *Metarhizium anisopliae*) as well as the interaction between species and treatment on the frequency of trophallaxis. * represent significance at $p < 0.05$, *** represent significance at $p < 0.001$ whilst lines crossing zero are NS.

Patterns were more distinct when assessing how species and treatment affected the duration of trophallaxis. Overall, we found that ants responded to both Tween (β: -0.11016, SE: 0.01087, z: -10.13, $p < 0.001$ see Figure 3.6 and Table S 3.4) and fungal challenge (β: -0.15980, SE: 0.01174, z: -13.62, $p < 0.001$ see Figure 3.6 and Table S 3.4) by engaging in shorter bouts of trophallaxis. *Tetramorium sericeiventre* also engaged in shorter bouts of trophallaxis compared to *A. custodiens* (β: -0.26421, SE: 0.12340, z: -2.14, $p = 0.032$ see Figure 3.6 and Table S 3.4). The significant interaction effects of species and treatment on the duration of trophallaxis is largely driven by the very short bouts within the first 48 hours (baseline data). *Camponotus fulvopilosus* engaged in longer bouts of trophallaxis compared to *A. custodiens* in both the Tween (β: 0.61994, SE: 0.01681, z: 36.87, $p < 0.001$ see Figure 3.6, Table S 3.4 and Figure 3.7 D for marginal effects of interactions) and fungal challenge (β: 0.70692, SE: 0.01791, z: 39.46, $p < 0.001$ see Figure 3.6, Table S 3.4 and Figure 3.7 D for marginal effects of interactions) treatments. Similarly, *T. sericeiventre* also engaged in longer trophallactic interactions compared to *A. custodiens* in both Tween (β: 0.54216, SE: 0.02203, z: 24.61, $p < 0.001$ see Figure 3.6, Table S 3.4 and Figure 3.7 D for marginal effects of interactions) and fungal challenges (β: 0.63796, SE: 0.02299, z: 27.75, $p < 0.001$ see Figure 3.6, Table S 3.4 and Figure

3.7 D for marginal effects of interactions). Mean and standard deviations for the frequency and duration of each behaviour across the three species and treatments were calculated (Table S 3.5).

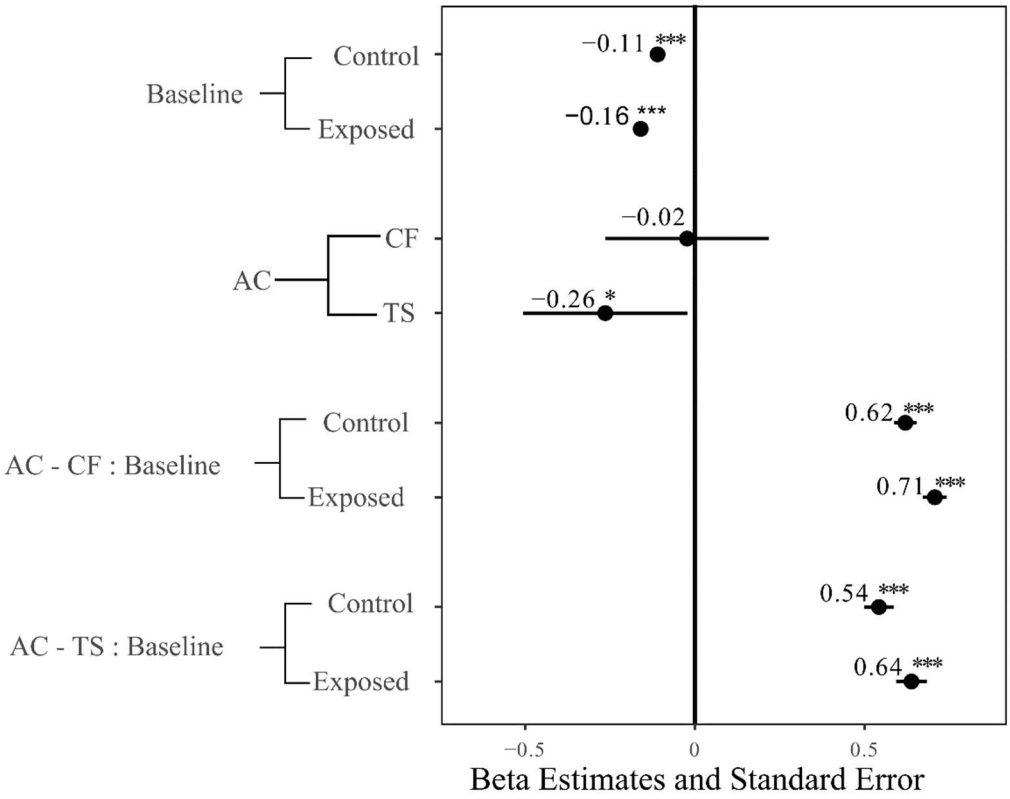

Figure 3.6: Forest plot of Beta estimates and standard error from a GLMM assessing the effects of species (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre*) and treatment (Baseline, Tween control or Exposed to *Metarhizium anisopliae*) as well as the interaction between species and treatment on the duration of trophallaxis. * represent significance at $p < 0.05$, *** represent significance at $p < 0.001$ whilst lines crossing zero are NS.

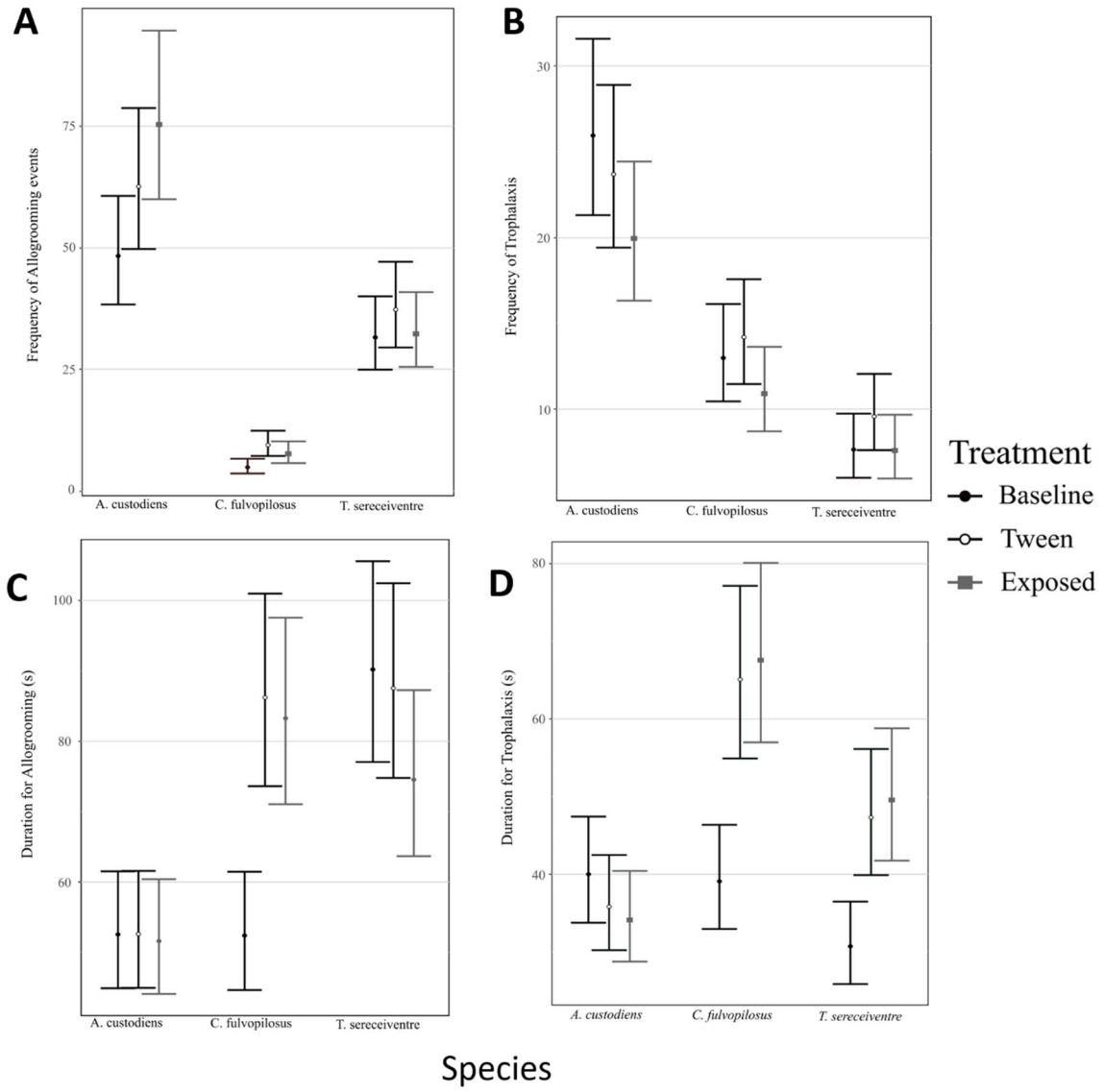

Figure 3.7: Plot of Marginal effects and 95% CI of the interaction between Species (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre*) and treatment (baseline, Tween control, Exposed to *Metarhizium anisopliae*) as well as the interaction between species and treatment on the A: frequency of allogrooming, B: frequency of trophallaxis, C: duration of allogrooming and D: duration of trophallaxis produced from GLMM predictions.

Discussion

Social immunity acts through interactions between individuals in response to infection (Cremer *et al.*, 2007). Reliance on these contact mechanisms are expected to vary across species (Bos *et al.*, 2019) and life histories (Walker & Hughes, 2011). In this study, response to pathogen exposure varied across the three South African ant species. Across all treatments, *A. custodiens* relied on frequent allogrooming interactions, whilst *T. sericeiventre* relied on lengthy allogrooming bouts. *Camponotus fulvopilosus* engaged in very little allogrooming. Species also differed in their response to treatments, with *A. custodiens* increasing the frequency of allogrooming when exposed to pathogens. *Tetramorium sericeiventre* decreased the duration of their allogrooming in response to pathogen exposure, while *C. fulvopilosus* increased the duration of allogrooming in response to both control substances and pathogen exposure. In response to treatment *A. custodiens* decreased the frequency and duration of trophallaxis, but *C. fulvopilosus* and *T. sericeiventre* increased the duration of trophallaxis in response to treatment with Tween or fungal conidia.

Anoplolepis custodiens interacted frequently in unmanipulated colonies, but these interaction rates were plastic, with colonies upregulating the frequency of allogrooming and downregulating the frequency of trophallaxis in response to Tween treatment and pathogen challenge. Increased allogrooming in response to pathogen challenge has been well documented (Walker & Hughes, 2009; Okuno *et al.*, 2012), and for some species this upregulation is independent of the pathogenicity of the treatment (Graystock & Hughes, 2011; Tranter *et al.*, 2015b). In our study we found that *A. custodiens* upregulates their frequency of allogrooming in response to the Tween20 treatment but further increased allogrooming rates in response to pathogen challenge; yet, the duration of allogrooming rates did not differ between treatments. It is worth noting here that the baseline treatment represents unmanipulated colonies whilst the Tween treated period represents the positive control. Our data show that the Tween elicits responses, which previous studies have not accounted for. Further, *A. custodiens* decreased their investment into trophallaxis in response to pathogen exposure, this finding has only been observed in one other study (Bos *et al.*, 2012), where *Camponotus aethiops* ants, exposed to *Metarhizium bruneum,* decreased all interactions with nestmates. Given the detected pattern of increased frequency of allogrooming and decreased frequency and duration of trophallaxis in response to pathogen challenge, it appears *A. custodiens* makes use of frequent short-term interactions to manage pathogen exposure by

focussing on allogrooming. Shorter bouts of allogrooming may minimise secondary transfer by decreasing the time at risk for each nestmate providing grooming; however, this has yet to be experimentally validated. Whilst trophallaxis may result in secondary transfer it does not facilitate the removal of conidia from exposed individuals and as such is expected to occur less frequently in the face of pathogen exposure.

In contrast to *A. custodiens, C. fulvopilosus* engaged in much fewer allogrooming and trophallactic events. *Camponotus fulvopilosus* workers marginally upregulated the frequency of allogrooming from baseline in response to treatments, although no change was detected between Tween and pathogen exposure. When *Lasius japonicus* ants were exposed to varying doses of *M. anisopliae* they increased the duration of allogrooming (Okuno *et al.*, 2012), suggesting that increased durations in allogrooming may aid in the management of pathogens, as was predicted by our model for *C. fulvopilosus* exposed to Tween and pathogen. We found that *C. fulvopilosus* responded to treatment by upregulating the duration of both allogrooming and trophallaxis, which suggests that allogrooming may not be the primary mechanism for pathogen control in *C. fulvopilosus*. This may be further strengthened by our findings (see Chapter 2: Figure 2.8), that allogrooming provides no significant reduction in conidia loads over self-grooming in *C. fulvopilosus.* Potential explanations for this may be reliance on a high degree of cuticular melanisation, which has been associated with investments in innate immunity (Barnes & Siva-Jothy, 2000). An alternate and more likely reason for the poor investment in allogrooming may be the use of potent antimicrobial secretions. *Camponotus fulvopilosus* is characterised by a proclivity to spray formic acid as a defence mechanism (Robertson & Zachariades, 1997) and formic acid and other forms of venom have been shown to act as potent antimicrobials (Tragust *et al.*, 2013; Tranter *et al.*, 2014; Brütsch *et al.*, 2017; Pull *et al.*, 2018). During our observations we noted colonies engaging in collective disinfection, where an individual groomed their acidopore releasing a small droplet of venom, causing most individuals in close proximity to also engage in acidopore grooming (see Figure S 3.1). This behaviour was not unique to *C. fulvopilosus* as it was also detected in *A. custodiens* (see Figure S 3.2). Colonies of *C. fulvopilosus* are well characterised by a potent odour, suggesting a more widespread application of formic acid than in *A. custodiens*. We found that although the frequency of trophallaxis decreased in response to pathogen exposure they engaged in longer bouts of trophallaxis as has been detected in other studies (de Souza *et al.*, 2008; Hamilton *et al.*, 2011). Additionally, we observed that trophallaxis occasionally occurred in conjunction with acidopore grooming, suggesting a potential for oral uptake of acidopore

secretions and transfer via trophallaxis. Oral uptake of acidopore secretions has been identified as a potential avenue for pathogen management in *Camponotus pennsylvanicus* (Hamilton *et al.*, 2011). Therefore, it appears that *C. fulvopilosus* may rely on chemical mechanisms over physical mechanisms such as allogrooming for controlling pathogen exposure.

In *T. sericeiventre* we found intermediate frequencies of allogrooming although these frequencies did not vary much across treatments. *Tetramorium sericeiventre* engaged in longer bouts of allogrooming when exposed to a pathogen yet decreased the duration of their trophallactic interactions. *Tetramorium sericeiventre* engaged in very few trophallactic interactions, but our model predictions suggest otherwise indicating increased duration of trophallactic events in response to treatments, in the same manner as *C. fulvopilosus*. It is possible that *T. sericeiventre* may be upregulating trophallaxis to facilitate the transfer of antimicrobials; however, unlike *C. fulvopilosus,* we did not detect frequent acidopore grooming. Our findings suggest that *T. sericeiventre* relies on infrequent but lengthy allogrooming to manage pathogen exposure. To our knowledge no study has assessed the effects of bout length on the efficacy of grooming, which represents a key avenue for ongoing research. Future research may benefit from fluorescence microscopy, where fluorescent tagged conidia could facilitate the quantification of conidia removal based on interaction rates. To date fluorescent tagged conidia have been used to show that ants maintained in groups had a higher rate of conidia removal than ants maintained alone (Qiu *et al.*, 2014) and these fluorescent tags may facilitate data collection to explore many more novel hypotheses.

The varied response of ants to Tween20 and that of fungal challenge with *M. anisopliae* emphasises the importance of positive and negative controls. The variation in species responses detected in this study together with similar research (Walker & Hughes, 2011; Tranter & Hughes, 2015; Tranter *et al.*, 2015b; a; Bos *et al.*, 2019), shows that species can manage exposure to pathogens through a range of mechanisms and this should be pursued in future research. This study, while limited to three species with no phylogenetic control, provides an important sub-Saharan perspective missing from SI research. Our study has further highlighted new avenues for future research. It has identified a potential novel behaviour in collective disinfection, observed in two species, which needs further research. This study identified the potential for the importance of trophallaxis as an avenue for SI research through the transfer of acidopore products (Hamilton et al., 2011) or alternate antimicrobials. Finally, given the potential trade-off detected in this study between frequency and duration of allogrooming, it

emphasises a possible duration effect in allogrooming efficacy. Future work should explore colony level responses to exposure and infection across more species that occur over wider ecological niches.

Research contributions

DJP, with aid from TCW, conceived and designed the experimental setup and analysis. DJP implemented the experimental setup with aid from Chris Du Toit in collecting and annotating observations of *C. fulvopilosus*. DJP collected, processed and analysed the data. DJP wrote the paper with input from TCW and CH.

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Chapter 4: Foragers do not change spatial use patterns in response to pathogen exposure: assessments of organisational immunity in three African ant genera.

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Abstract:

Ant colonies are characterised by complex structural and behavioural patterns with individuals utilising a closed dense nest space, which exacerbates the risk of pathogen transmission. Ants utilise a range of behaviours termed social immunity and utilise variation in colony organisation, termed organisational immunity, to limit the impact of pathogens. Of particular interest in organisational immunity is how nestmates use space to limit pathogen risks prophylactically and in response to exposure. We assessed organisational immunity through analyses of nest space use across three species of South African ants, *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre* in response to exposure by the generalist entomopathogenic fungi *Metarhizium anisopliae.* All three species showed a high degree of clustering, regardless of pathogen exposure, which is expected to limit pathogen spread through colonies by restricting the pathogen to small groups; however, only one species, *A. custodiens,* showed strong patterns of organisational immunity. They kept their foragers, which are most likely to encounter pathogens, separate from the queen and brood and further responded to pathogen exposure by increasing clustering in the nest and having nest workers congregate nearer to the queen. In all three species, we noted that foragers did not alter their spatial use in response to infection suggesting they most likely utilise alternate mechanisms to manage pathogen risk between themselves and other nest members. Only one of the three species displayed prophylactic and activated organisational immunity, whilst the remaining two species relied solely on clustering.

Intended for submission to "Insectes Sociaux"

Introduction

The evolutionary transition from a solitary lifestyle to group living represents an important and impactful shift in the evolution of complexity (Szathmary & Maynard Smith, 1995). This transition facilitated eusociality in insects, which has allowed them to diversify and occupy many ecological niches across the globe (Wilson, 1971). Social insects partition their tasks and benefit from living in structured colonies, separating reproduction, maintenance, protection and resource acquisition (Wilson, 1985). In contrast, eusociality introduces new challenges of increasing pathogen risks and epidemics due to the dense, closely related individuals interacting within the nest (Schmid-Hempel, 1998). In order to combat these risks, social insects express group-level adaptations and behaviours which benefit the colony at a potential cost to the individual, termed social immunity (SI; Cremer *et al.*, 2007).

Within the suite of SI behaviours, of particular interest in colony-level prevention of pathogen spread is organisational immunity (OI; Naug & Smith, 2007). Organisational immunity is characterised as the properties of a colony's organisational structure, which limits the spread of pathogens within the colony and protects valuable individuals, namely the reproductives and the young within the society (Naug & Smith, 2007; Stroeymeyt *et al.*, 2014). The mechanisms of OI develop from spatial and behavioural compartmentalisation of individuals within the colony, through advanced task partitioning (Ratnieks & Anderson, 1999) and division of labour (Cremer *et al.*, 2007). Age polyethism, which facilitates division of labour and task partitioning, acts by assigning workers tasks according to their age, whilst caste polyethism partitions tasks by caste such as soldiers and minims (Griffiths & Hughes, 2010). Newly eclosed workers are assigned to queen and brood maintenance; as they age they move on to nest maintenance, then foraging, and often culminate in waste management (Seeley, 1982). This system ensures that perilous tasks, with greater chances for infection by pathogens (Ballari *et al.*, 2007; Fefferman *et al.*, 2007; Cremer & Sixt, 2009), are undertaken by older less valuable members of the colony, at the end of their lifespan. Task partitioning ensures that interaction occurs primarily between members engaging in the same tasks (groups) with certain individuals acting as bridges between these task groups creating spatial segregation within the colony (Naug & Smith, 2007). Further, this partitioning generates clustering (Salathe & Jones, 2010) and spatial heterogeneities, upon which OI can act.

Spatial-based epidemiological models have shown that increasing nest complexity and spatial structuring can limit pathogen spread rates (Hagenaars *et al.*, 2004; Pie *et al.*, 2004; Lindholm & Britton, 2007). Proximity networks implemented in the harvester ant *Pogonomyrmex*

barbatus showed that interaction rates were increased near the entrance (Pinter-Wollman *et al.*, 2011), likely to limit pathogen ingress. More recent advances in computation have increased the resolution with which spatial use has been assessed, by increasing tracking times as well as increasing the number of individuals that can be tracked simultaneously. In the ant *Camponotus fellah,* using video data, a complex barcoding identification system and tracking algorithm, the authors analysed 9,363,100 interactions and found that colony members can be broadly divided into three major groups based on their behaviours and interactions: the queen and her retinue, nest maintenance workers and foragers (Mersch *et al.*, 2013). Similarly, in a recent study on honeybees that combined social and spatial assessments of colony organisation, similar patterns of compartmentalised structuring were detected (Baracchi & Cini, 2014). Baracchi & Cini (2014) observed strong within group and weak between group overlap in interaction and spatial use of age groups (Baracchi & Cini, 2014). The queen was separated from foragers and middle-aged workers via temporal differences in the utilization of space or in the case of co-occurrence, separation was maintained by her retinue of young workers (Baracchi & Cini, 2014). These behaviours work together to generate clustering in spatial use with individuals associated with tasks utilising the same space and therefore limiting the potential spread of pathogens between groups. This spatial segregation or clustering is thought to decrease the exposure risk of high value individuals (Cremer *et al.*, 2007; Stroeymeyt *et al.*, 2014) and may even help limit secondary transfer of pathogens.

Organisational immunity behaviours occur either in a prophylactic context or can be activated when faced with an infection (Stroeymeyt *et al.*, 2014). Prophylactic OI includes modifications to colony structuring, which enhance interaction heterogeneities (Mersch *et al.*, 2013; Quevillon *et al.*, 2015; Stroeymeyt *et al.*, 2014). These modifications alter the pathways along which pathogens may spread and can be implemented spatially, temporally, or as behavioural adjustments (Stroeymeyt *et al.*, 2014, 2018). Examples include ostracising risky waste workers from the main nest regions (Hart & Ratnieks, 2001, 2002) or spatial separation of task groups with risky foragers located further from the queen and nest centre (Mersch *et al.*, 2013; Quevillon *et al.*, 2015). Activated responses are triggered upon the detection of pathogens, altering interaction networks. Examples of activated OI may include alarm behaviours that restrict contact between infected and non-infected individuals (Rosengaus *et al.*, 1999; Myles, 2002), the self-removal of infected nestmates (Chapuisat, 2010; Heinze & Walter, 2010; Bos *et al.*, 2012) or altering the degree of mobility in immune challenged individuals to facilitate spatial segregation (Aubert & Richard, 2008). Infected ants have even been shown to stop

tending important individuals such as brood and queens (Ugelvig & Cremer, 2007; Bos *et al.*, 2012).

One aspect of consideration in spatial use within the nest is the potential avenue of "same place different time" interactions which may facilitate the transfer of potential pathogens in the nest. In a study of *Temnothorax albipennis*, the authors assessed how spreading agents that persist in the local environment (i.e. fungal conidia) may alter transmission within the colony (Richardson & Gorochowski, 2015). They found that the implementation of indirect pathways of spread via same place different time interactions resulted in altered spread rates dependent on the modelled longevity of the agent (Richardson & Gorochowski, 2015). Pathogens may spread through physical contact with a nestmate but can also spread indirectly through shared nest space (Fries & Camazine, 2001; Otterstatter & Thomson, 2007). Further, spatial use influences interaction likelihoods (Krause *et al.*, 2009), through modifying proximity, consequently playing an important role in mitigating disease. Many studies have detected more spatial segregation in work groups with foragers utilising different nest spaces than the reproductive and brood (Sendova-Franks *et al.*, 2010; Mersch *et al.*, 2013; Baracchi & Cini, 2014; Quevillon *et al.*, 2015). To date most studies assessing OI and spatial responses in colonies have not included pathogen exposure (but see, Stroeymeyt *et al.*, 2018) and thus may miss induced OI. Further, to our knowledge, spatial use patterns have not been assessed across species of ants, particularly in response to pathogen exposure. Given the frequency of detected spatial heterogeneities in nests (Naug, 2008; Mersch *et al.*, 2013; Quevillon *et al.*, 2015), and their potential importance in limiting pathogen spread (Stroeymeyt *et al.*, 2014), this study assesses spatial use patterns across three African ant species in response to pathogen exposure.

We assessed spatial use patterns, specifically assessing general spatial use, distance to the queen and overall colony clustering in three South African ant species. The three species assessed were *Anoplolepis custodiens*, an ecologically and numerically abundant species (Samways, 1990), *Camponotus fulvopilosus*, a xeric adapted species which frequently employs formic acid for defence (Robertson & Zachariades, 1997) and *Tetramorium sericeiventre* which represents a species with broad ecological and environmental niches that has been shown to co-occur with invasive Argentine ants (Luruli, 2007). We expected that all species would exhibit prophylactic organisational immunity in the form of spatial segregation between queens and foragers (Mersch *et al.*, 2013). Further we expect that species will respond to pathogen exposure, upregulating their OI, by increasing the degree of clustering and decreasing within-

nest worker distance to the queen. Foragers that are infected are expected to decrease their use of nest-space and cluster to a greater degree and increase their distance to the queen to limit potential avenues of secondary transfer of pathogens.

Methods

Experimental setup

Three colonies each were collected for *A. custodiens, T. sericeiventre* and *C. fulvopilosus.* All colonies were maintained as per Chapter two. We made use of the generalist insect entomopathogen *Metarhizium anisopliae* (META69, isolate: ICIPE69) for all pathogen exposures given its frequent use in studies of social immunity (Traniello *et al.*, 2002; Hughes & Boomsma, 2004a; Pie *et al.*, 2005; Reber & Chapuisat, 2012; Tragust *et al.*, 2013b; Tranter *et al.*, 2015; Qiu *et al.*, 2016). We used the methodology described in Chapter two for fungus cultivation, preparation and application. Preliminary assessments of mortality rates resulting from the exposure differed between species (*A. custodiens*: 85%, *C. fulvopilosus:* 83% and *T. sericeiventre:* 50%) and of all dead ants, only ~9% had not succumbed to *Metarhizium anisopliae* infections.

Experimental nests were set up for each collected colony as per Chapter three, with colonies housed between two anti-reflective glass panels separated by JoinTech and attached to a foraging arena. Available nest space was proportional to average nest-worker sizes; in *A. custodiens* (±800 mm^2 X 5 mm height, Figure 3.1 A), *C. fulvopilosus* (±1300 mm^2 X 10 mm height, Figure 3.1 B) and *T. sericeiventre* (±500 mm^2 X 3 mm height, Figure 3.1 C). Nests were kept in dark filming boxes and recorded under infrared illumination (Figure 3.2). Foraging arenas were exposed to a temperature (± 25 ºC) and humidity-controlled environment with a 12-hour day night cycle, further colonies were provided ad libitum access to sugar water and a protein source in the form of termites every three days.

Three experimental colonies per species were set up with 100 worker ants, a queen and a mixture of brood, eggs and pupae (AC: ±1.5 cm^2, CF: ±3 cm^2 and TS: ±1 cm^2). As per Chapter three, experimental nests were allowed a 24-hour acclimation phase, during which any foragers (present in the arena) were marked (POSCA PC-5 marker) on their abdomen. Forager checks were repeated three times daily for the duration of the trial to identify and mark any new foragers in the arena. We then treated 10 foragers per colony with a 5% Tween 20 solution, as per previous protocol (Chapter 1), and marked their thorax, hereafter termed “Tween” foragers. Tween foragers were returned to the foraging arena and allowed to reintegrate for 24-hours.

Colonies were maintained for 48 hours before repeating the process by exposing a further 10 foragers to *Metarhizium anisopliae*. Foragers were treated with a 1 x 10^7 conidia per ml Tween 20 solution (5%), as per previous protocol (Chapter 1). Treated foragers are hereafter termed "exposed" foragers. Exposed foragers were marked on their head and returned to the foraging arena, after a 24-hour reintegration period we maintained colonies for a final 48 hours. A snapshot of spatial use within the colony was taken every four hours during each 48-hour window. This resulted in 12 snapshots collected per colony and treatment (Tween and Pathogen exposure) for each species, resulting in a total of 72 snapshots per species collected for subsequent analysis. For each snapshot, using WebPlotDigitizer (Rohatgi, 2019) we determined XY coordinates for each individual within the colony as well as the location of brood. Further we identified the functional group of each individual (Queen, Nest-worker, Brood, or Forager) and the status of the foragers (Untreated, Tween treated or Pathogen exposed).

Statistical Analysis

All analyses were completed in the R studio statistical environment. Data analyses were conducted using the spatstats package (Baddeley *et al.*, 2015) in R. In order to visualise spatial use patterns, we generated maps for each colony during the *M. anisopliae* exposure phase, based on individual functional group and forager status. This was accomplished by computing kernel density estimates over the pathogen treatment period for each colony, which calculates an estimate of density across the available space for individuals of a given functional group or forager status. We further calculated nearest neighbour Euclidean distances to the queen for each individual per snapshot. Finally, for each snapshot, we implemented the Clark-Evans test of aggregation for a spatial point pattern (Clark & Evans, 1954), to estimate the degree of clustering or ordering in spatial use. Clark-Evans aggregation index values above one indicates an ordering of spatial use whilst values below one indicates a clustered pattern (see Figure 4.1). We utilised the edge correction of Donnelly (1978) to account for any potential edge effects. In order to assess whether foragers shift their spatial use in response to infection we compared the Euclidean distance to queens of untreated and pathogen-exposed foragers during the infection treatment using a Mann-Whitney U test. Secondly, we compared how nest workers change their Euclidean distance to the queen in response to treatment by comparing nest workers from the Tween treatment to the infection treatments using a Mann-Whitney U test. Finally, we compared colony level Clark and Evans scores (degree of clustering) across

treatments using a Wilcoxon signed rank test to determine how Tween and pathogen exposure affect colony clustering. All analyses were run separately for each colony.

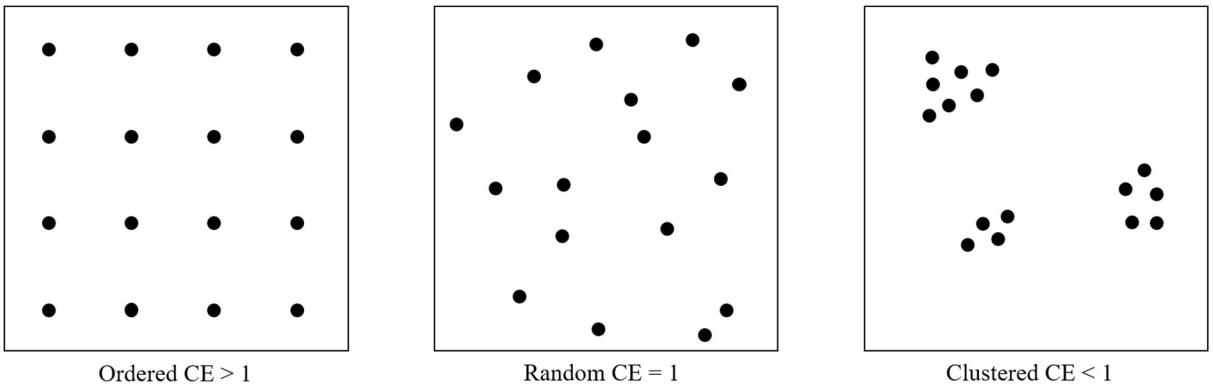

Figure 4.1: Graphical representation of Clark and Evans aggregation scores, with scores above one depicting ordered spatial use, values of one representing random spatial use and values below 1 representing clustered spatial use.

Results

Spatial use across all colonies of *A. custodiens* showed distinct spatial segregation between the queen with her brood and foragers regardless of their status (Untreated, Tween treated, or Pathogen exposed). Nest-workers co-occurred with both groups, likely acting as a bridge for communication. This pattern held for all three colonies across both treatments (Figure 4.2, only infection trials shown); yet, foragers did not alter their spatial use based on status with untreated, Tween and pathogen exposed foragers all co-occurring in space (Figure 4.2). Such clear spatial separation was not present in *C. fulvopilosus* (Figure 4.3), with overlap between foragers, nest-workers and the queen. In a single colony (Colony 2), infected foragers were separated in space from the queen (Figure 4.3 B), while in the other colonies overlap occurred to a greater degree (Figure 4.3 A and C). Finally, *T. sericeiventre* showed a very low degree of spatial segregation, with all individuals overlapping in their spatial use patterns, with no discernible separation (Figure 4.4).

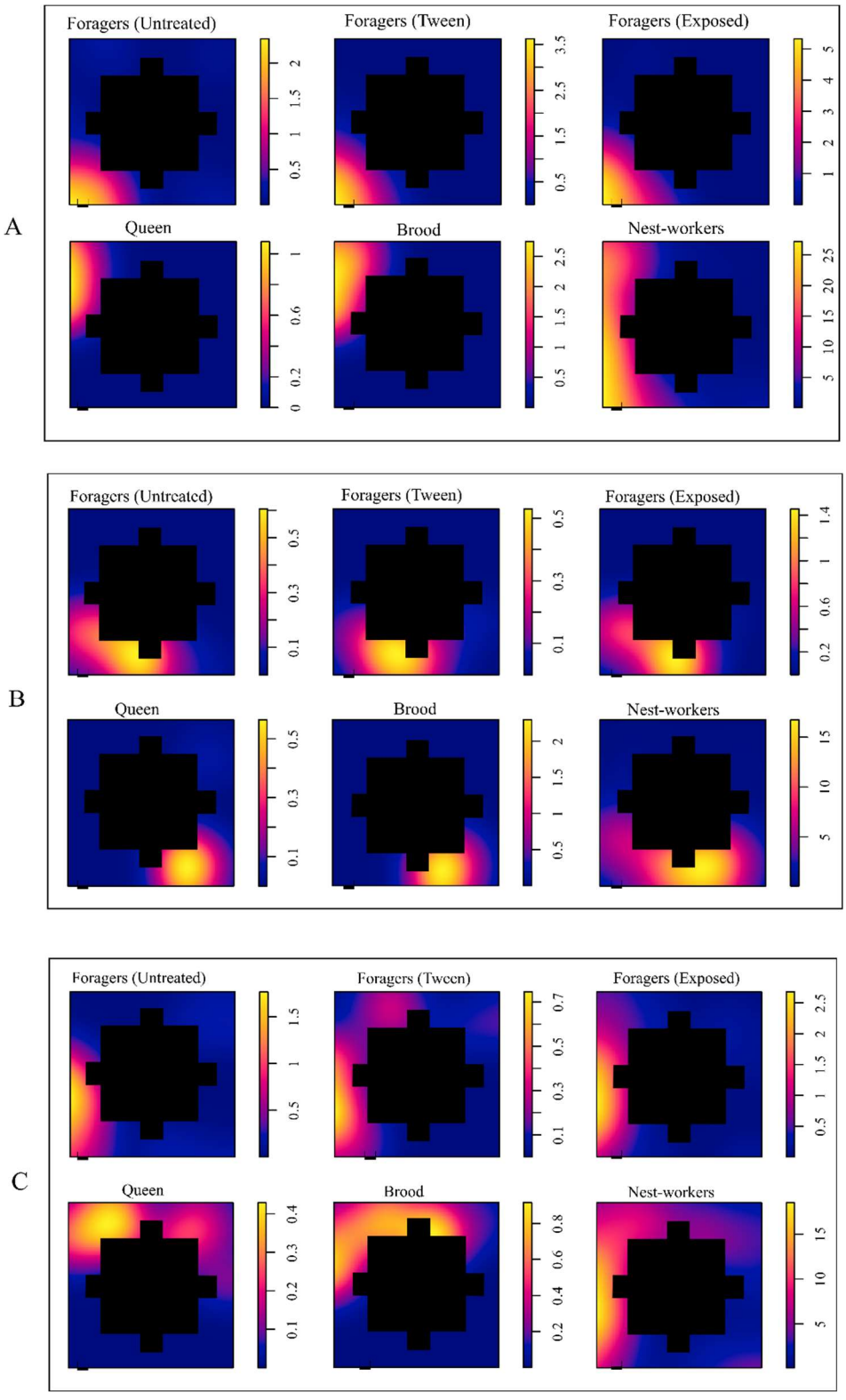

Figure 4.2: Heatmaps of spatial use patterns in *Anoplolepis custodiens* in the pathogen exposure treatment. Warmer colours represent a higher estimated density of individuals occurring in space whilst cooler colours represent lower estimated densities, calculated from kernel density estimates. Density estimates were generated for each status (queen, brood, nest-workers, untreated foragers, Tween foragers or exposed foragers) separately. A-C represent results from Colony 1-3 respectively. Black overlay represents inaccessible space and the nest entrance is depicted in black in the bottom left of nest.

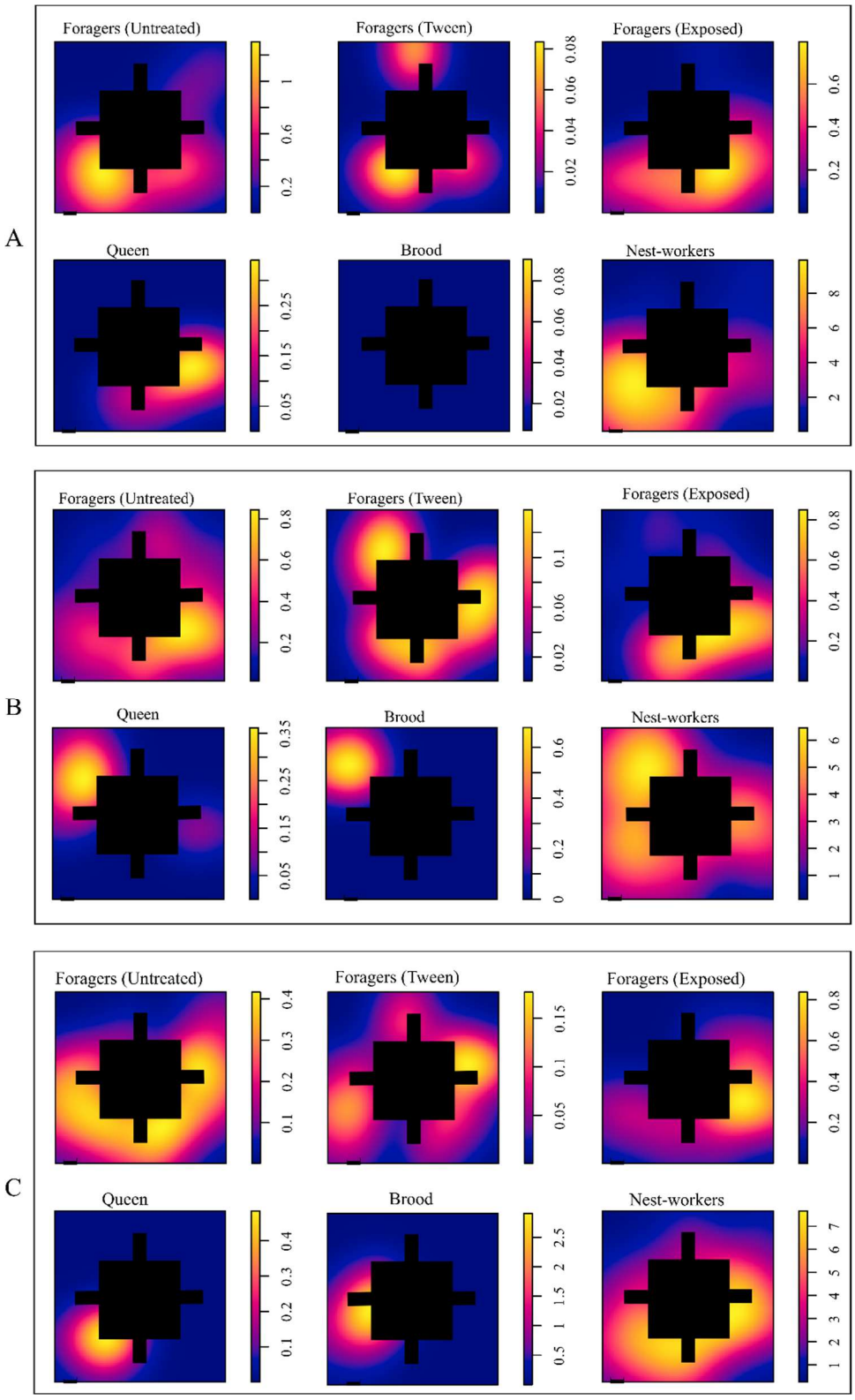

Figure 4.3: Heatmaps of spatial use patterns in *Camponotus fulvopilosus* in the pathogen exposure treatment. Warmer colours represent a higher estimated density of individuals occurring in space whilst cooler colours represent lower estimated densities, calculated from kernel density estimates. Density estimates were generated for each status (queen, brood, nest-workers, untreated foragers, Tween foragers or exposed foragers) separately. A-C represent results from Colony 1-3 respectively. Black overlay represents inaccessible space and the nest entrance is depicted in black in the bottom left of nest.

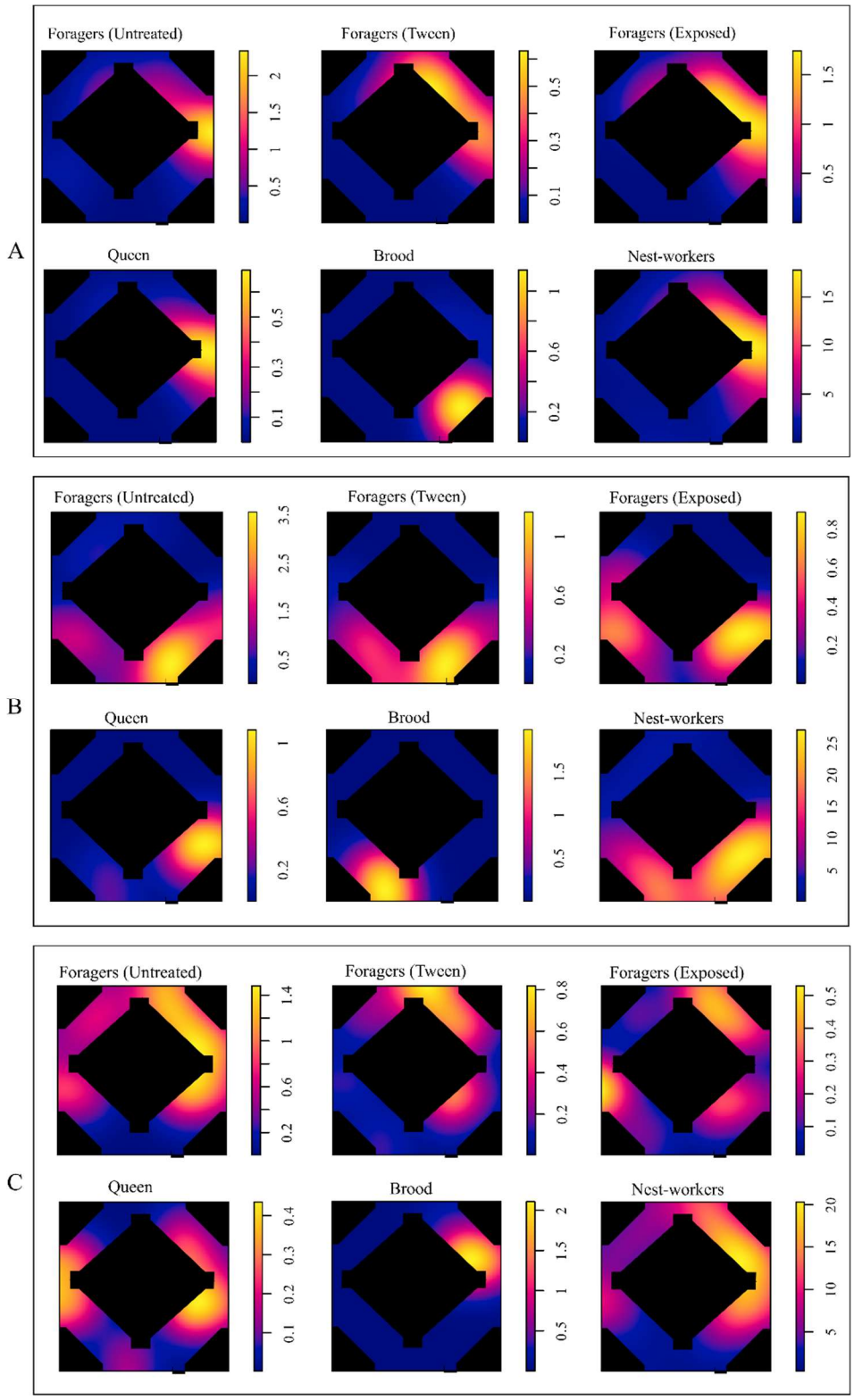

Figure 4.4: Heatmaps of spatial use patterns in *Tetramorium sericeiventre* in the pathogen exposure treatment. Warmer colours represent a higher estimated density of individuals occurring in space whilst cooler colours represent lower estimated densities, calculated from kernel density estimates. Density estimates were generated for each status (queen, brood, nest-workers, untreated foragers, Tween foragers or exposed foragers) separately. A-C represent results from Colony 1-3 respectively. Black overlay represents inaccessible space and the nest entrance is depicted in black in the bottom right of nest.

Foragers did not alter their Euclidean distances to the queen after exposure to the pathogen, except for *A. custodiens* foragers, from colony 3 (Figure 4.5 C), which decreased their distance to the queen compared to untreated foragers (W = 1126, p = 0.046). *Anoplolepis custodiens* nest-workers spent time closer to the queen in pathogen exposed treatments compared to the Tween treatments for two of the colonies (Colony 1: W = 289256, p < 0.001, Figure 4.6 A; Colony 2: W = 226642, p < 0.001, Figure 4.6 B). Nest-workers in two colonies of *C. fulvopilosus* showed significant changes in distance to the queen, with one colony increasing distance in response to pathogen exposure (Colony 1: W = 295856, p < 0.001, Figure 4.6 D) and another decreasing their distance (Colony 2: W = 251112, p = 0.030, Figure 4.6 E). Nest-workers of a single colony of *T. sericeiventre* significantly increased their distance to the queen in response to pathogen exposure (Colony 3: W = 269271, p < 0.001, Figure 4.6 I). All calculated Clark and Evans scores were below one, suggesting that all three species exhibited clustering regardless of treatment (Figure 4.7). When comparing how treatment affected Clark and Evans scores, we found that only two colonies of *A. custodiens* responded to pathogen exposure by increasing the degree of their clustering (Colony 1: V = 12, p = 0.034 Figure 4.7 A; Colony 3: V = 4, p = 0.034 Figure 4.7 C).

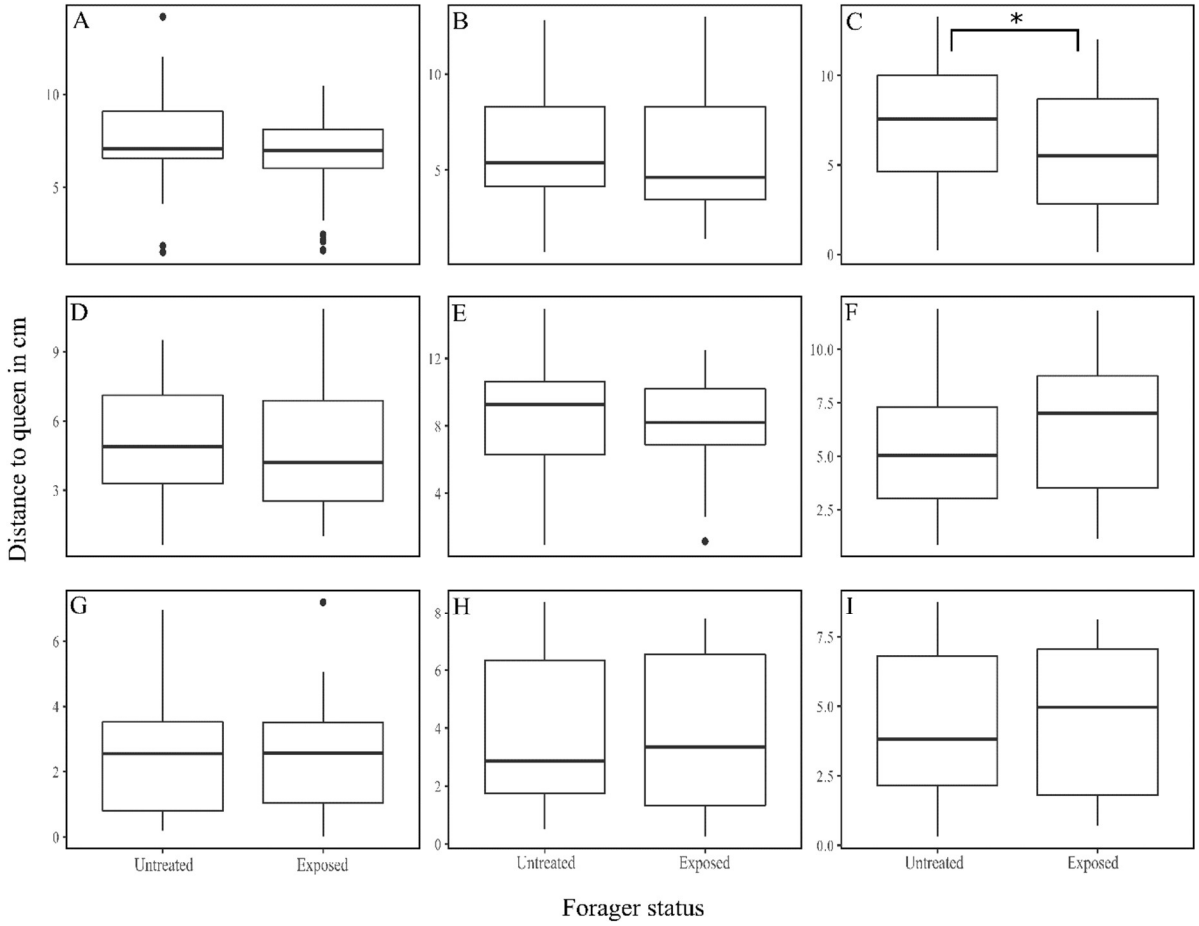

Figure 4.5: Boxplots depicting the median Euclidean distance to queen (cm) for untreated and exposed foragers in the pathogen exposure treatment. A-C represent colonies 1-3 of *Anoplolepis custodiens*, D-F represent colonies 1-3 of *Camponotus fulvopilosus* and G-I represent colonies 1-3 of *Tetramorium sericeiventre* respectively. * mark significant differences based on Mann-Whitney U tests at $p < 0.05$. Boundaries of boxes indicate the first and third quartiles, the solid line represents medians, whiskers represent 1.5 times the interquartile range and dots represent outliers.

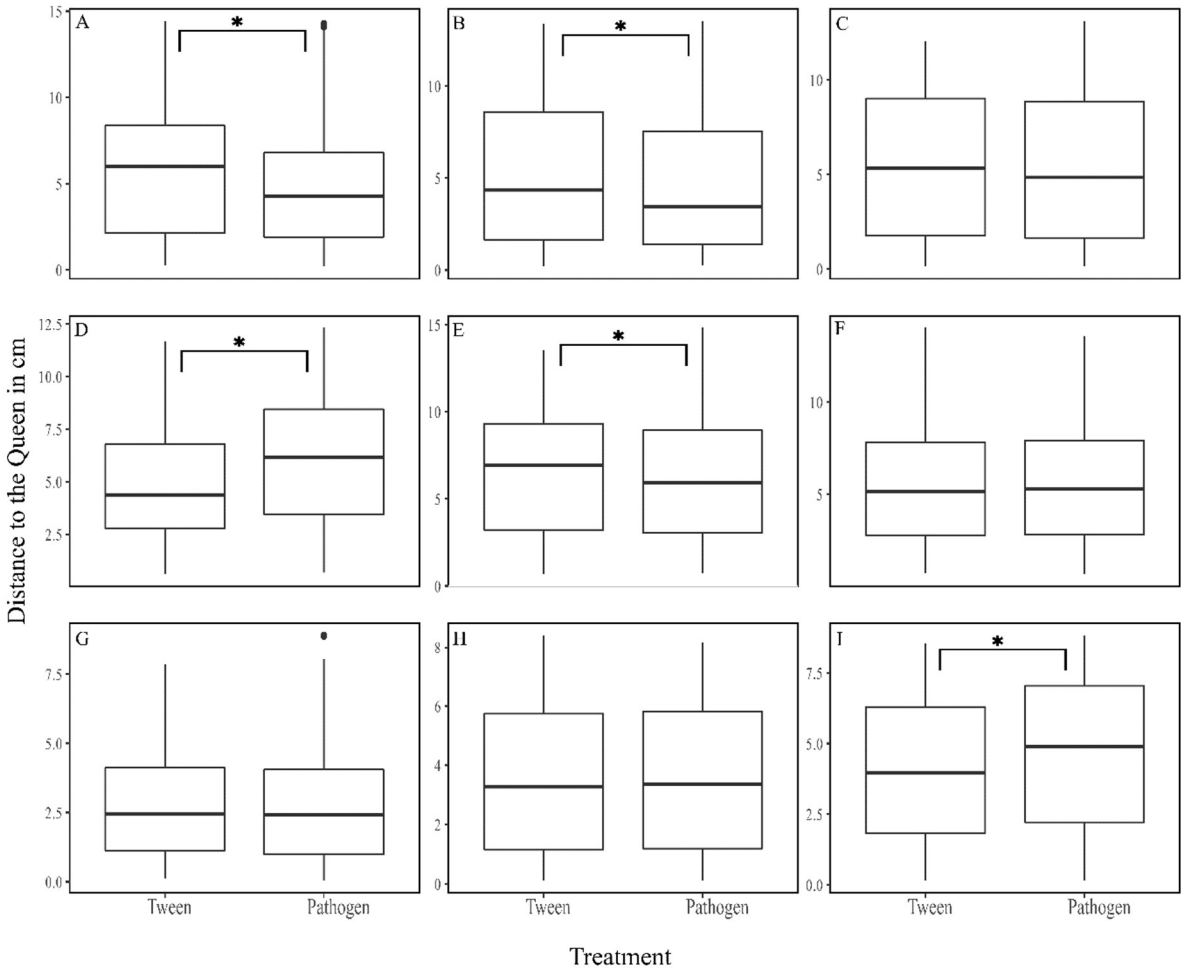

Figure 4.6: Boxplots depicting the median Euclidean distance to queen (cm) for nest-workers in the Tween and pathogen exposure treatments. A-C represent colonies 1-3 of *Anoplolepis custodiens*, D-F represent colonies 1-3 of *Camponotus fulvopilosus* and G-I represent colonies 1-3 of *Tetramorium sericeiventre* respectively. * mark significant differences based on Mann-Whitney U tests at $p < 0.05$. Boundaries of boxes indicate the first and third quartiles, the solid line represents medians, whiskers represent 1.5 times the interquartile range and dots represent outliers.

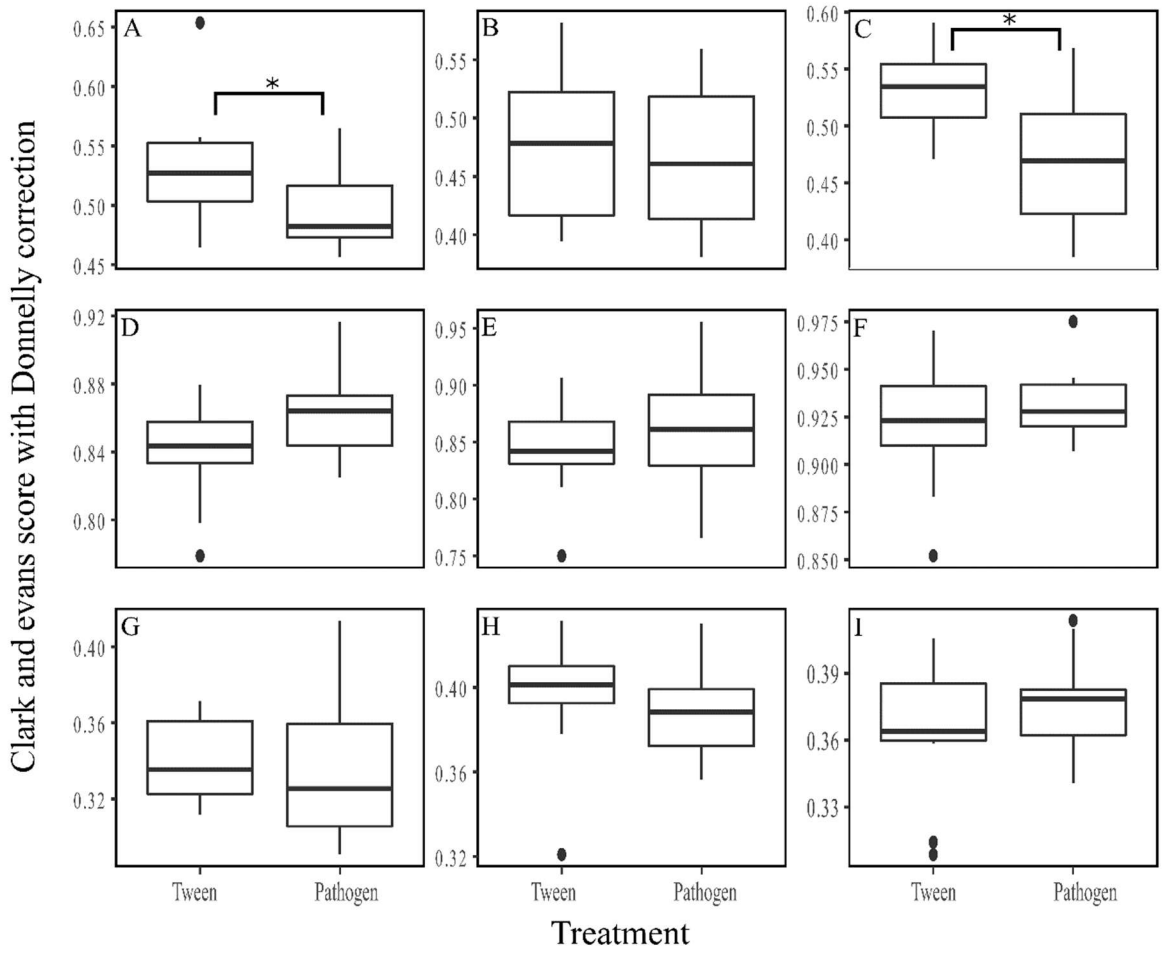

Figure 4.7: Boxplots depicting the median Donnelly adjusted Clark and Evans scores for colonies in Tween and pathogen exposure treatments. A-C represent colonies 1-3 of *Anoplolepis custodiens*, D-F represent colonies 1-3 of *Camponotus fulvopilosus* and G-I represent colonies 1-3 of *Tetramorium sericeiventre* respectively. * mark significant differences based on Wilcoxon signed rank test at $p < 0.05$. Boundaries of boxes indicate the first and third quartiles, the solid line represents medians, whiskers represent 1.5 times the interquartile range and dots represent outliers.

Discussion

Prophylactic and activated OI were identified in only one of the three species assessed in this study. *Anoplolepis custodiens* showed clear spatial segregation with little overlap in spatial use between foragers and the queen with her brood regardless of treatment status. This finding is strongly indicative of prophylactic OI. Furthermore, in response to pathogen exposure, *A. custodiens* increased their clustering and nest-workers decreased their distance to the queen, suggesting activated OI; however, no patterns of OI were detected for *C. fulvopilosus* and *T. sericeiventre*. Across all three species we found that forager spatial use patterns remained consistent regardless of pathogen exposure in all but one colony of *A. custodiens*, suggesting that ants do not alter their spatial use in response to infection.

Spatial segregation represents a primary form of prophylactic OI (Hughes & Cremer, 2007; Stroeymeyt *et al.*, 2014; Cremer *et al.*, 2018), whereby important individuals are protected through spatial use heterogeneities. In a study on *Camponotus fellah,* Mersch *et al.* (2013) identified spatial segregation between foragers, nurses and nest cleaners, although their study did not introduce any form of pathogen. In a more recent assessment of social and spatial use in an ant species, Stroeymeyt *et al.* (2018) showed similarly that foragers, which are expected to encounter pathogens, segregated from young workers and queens. Further, using simulations, they showed that this organisation disproportionally protects high-value individuals from pathogen challenge (Stroeymeyt *et al.*, 2018). This has further been corroborated by the inclusion of spatial segregation in models assessing infection dynamics showing decreased pathogen spreading (Pie *et al.*, 2004). Our study found patterns of spatial segregation between foragers and the queen in *A. custodiens,* suggesting they employ prophylactic OI, as this pattern occurred across both treatments. Spatial segregation is expected to minimise contact rates between high value and risky individuals (Stroeymeyt *et al.*, 2014) whilst further limiting the likelihood of secondary pathogen transfer through shared use of physical space, as has been suggested by Richardson & Gorochowski (2015). The costs incurred by spatial segregation is a decrease in information and resource flow through the colony (Sendova-Franks *et al.*, 2010; Blonder & Dornhaus, 2011); yet, the benefits accrued through protection against pathogen challenge may outweigh the costs.

Spatial segregation may also be induced by pathogen challenge, leading to changes in the degree of clustering and to changes in nestmate distances to high-value individuals. We detected activated organisational immunity in *A. custodiens* in response to pathogen exposure. Nest-workers of *A. custodiens* decreased their distance to the queen and the colony overall, increased the degree of clustering in response to pathogen exposure. This suggests that nest workers clustered closer to the queen and her brood, creating a barrier to limit interactions with exposed foragers. Research in honeybees showed that the queens retinue prevent any direct interaction between the queen and foragers, despite spatial and temporal overlap in nest use (Baracchi & Cini, 2014). Similar findings were reported for *Lasius niger*, where nest-workers increased their spatial overlap with brood in response to infection and increased their clustering (Stroeymeyt *et al.*, 2018). These shifts in clustering and spatial use in response to infection are indicative of activated organisational immunity where colonies employ behavioural shifts to limit the potential spread of detected pathogens. Clustering in simulated communities has been shown to limit disease spread in computational models (Wu & Liu, 2008). In particular when clustering occurs, disease outbreaks are limited to within groups and overall pathogen prevalence is decreased (Nunn *et al.*, 2015). Nest-workers of *A. custodiens* reduced their distance to the queen, indicating that colonies actively respond to infection by augmenting their prophylactic spatial segregation; however, this is driven by nest workers as distance to the queen did not change in foragers regardless of exposure status.

Pathogen detection is well documented in ants (Cremer *et al.*, 2007; Reber *et al.*, 2011; Diez *et al.*, 2015; Stroeymeyt *et al.*, 2018) and as such, activated OI was expected. Furthermore, multiple studies have shown that individuals react to their infection by isolating themselves from nestmates (Heinze & Walter, 2010; Bos *et al.*, 2012), by distancing themselves from the queen and brood chambers (Ugelvig & Cremer, 2007) or even increasing the proportion of time spent foraging (Stroeymeyt *et al.*, 2018). Thus, we expected foragers to alter their spatial use patterns when exposed to pathogens. We did not find a clear shift in forager proximity to the queen, in response to exposure across all three species. A likely explanation is that these changes in behaviour may be related to infection rather than exposure, *Metarhizium anisopliae* takes between 12 and 24 hours for conidia to germinate and pierce the cuticle of the forager and for infection to occur (Hajek & Leger, 1994; Moino *et al.*, 2002). We assessed spatial patterns within 48 hours of exposure during which the exposed foragers may not have become infected, or their infection was still developing. It has been shown that on the third day post infection *Myrmica rubra* ants showed decreased attraction to nestmates or their odour (Leclerc

& Detrain, 2017); however, the proximate reasons for this are still under debate. Alternatively, the risk posed by foragers exposed to pathogens is weighed against the beneficial resources they bring into the nest and, rather than limiting forager access to the nest, colonies rely on alternate mechanisms for pathogen control for example grooming (Zhukovskaya *et al.*, 2013). Finally, it is possible that our Euclidean distance measures may not accurately represent walking distances within the colony.

Our study only identified patterns of OI in one of the studied species. *Anoplolepis custodiens* presented both prophylactic and induced changes in spatial use patterns; but, the same could not be said for *C. fulvopilosus* and *T. sericeiventre*. This raises the question of what drives differential investments into organisational immunity. One possibility is that species are reliant on alternate prophylactic mechanisms of pathogen control. In particular, *C. fulvopilosus* is characterised by a proclivity to spray formic acid (Robertson & Zachariades, 1997) and could rely on formic acid as an anti-microbial (Tragust *et al.*, 2013; Tranter & Hughes, 2015; Brütsch *et al.*, 2017) in lieu of OI. Alternatively, interaction with exposed individuals may facilitate the transfer of low-level infections which may facilitate colony health through immune priming (Konrad *et al.*, 2012; Liu *et al.*, 2015). Whilst OI was not detected in two of the three species, based on spatial use patterns, it may be possible that OI is maintained on the basis of interaction heterogeneities as was detected in *Camponotus pennsylvanicus* (Quevillon *et al.*, 2015). Similarly, Quevillon *et al.* (2015) found that spatial use patterns did not differ greatly between foragers, nest-workers or queens. Yet, when assessing the timing and order of trophallaxis events they found that foragers did not interact in a manner that would facilitate transfer of potential pathogens to the queen, as they always made use of nest-workers as brokers (Quevillon *et al.*, 2015). This highlights the importance of assessing OI both spatially, temporally and behaviourally.

It is worth noting that this study included intact artificial nests with brood, a queen and workers. However, in order to quantify behavioural interactions workers, we were confined to 100 individuals and whilst our findings are unlikely truly representative of natural behaviour, our detection of OI in one species suggests that collective behaviour can develop in small groups. Our study assessed spatial use over a limited period before the onset of infection and as such activated OI may be more readily observable in the face of an epidemic or as infections have time to establish. Thus, future work should extend the period of data collection post exposure. Future work may benefit from assessing both Euclidean distances and path distances within

the nest when calculating nearest neighbour distances to the queen. A further consideration in future studies would be to assess locomotion in healthy and infected nest workers as previous work has shown that movement patterns may also play a role in generating interaction heterogeneities (Aubert & Richard, 2008; Pinter-Wollman *et al.*, 2011), through which OI mechanisms primarily act. Assessments of waste management in future studies assessing OI would be beneficial given the pathogen risk of the behaviour, together with the wealth of behavioural adaptations associated with waste management (Hart & Ratnieks, 2002; Renucci *et al.*, 2011; Diez *et al.*, 2012, 2014).

Our study identified both prophylactic and activated OI in one of three assessed South African ant species, highlighting the importance of multispecies comparisons. We found that *A. custodiens* showed spatial segregation between foragers and the queen with her brood. Further, in response to infection they increased their clustering and nest-workers converged around the queen. When assessing spatial use shifts in foragers we found that across all three species, no changes occurred in response to exposure to *M. anisopliae*. Finally, our findings emphasise the importance of assessing pathogen control across a range of social immunity mechanisms, as each species appears to rely on different behavioural adaptations to manage infections.

Research contributions

DJP, with aid from TCW, conceived and designed the experimental setup and analysis. DJP implemented the experimental setup with aid from Chris Du Toit in collecting observations of *C. fulvopilosus*. DJP collected, processed and analysed the data. DJP wrote the paper with input from TCW and CH.

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Chapter 5: Changes in interaction networks in *Camponotus fulvopilosus* in response to pathogen exposure

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Abstract:

Social insect colonies are characterised by a large network of interacting individuals acting for the benefit of the colony. These interactions facilitate the spread of information and resources. However, interactions can also spread pathogens throughout the social network. Ants are expected to manage their interaction patterns by engaging in social immune behaviours to facilitate continued colony functioning whilst limiting potential infections. Network analyses allow for the assessment of interactions and through a range of metrics can characterise the structure of the social network. Using the South African ant species, *Camponotus fulvopilosus*, we observed all trophallactic and allogrooming interactions that occurred during a four-day Tween control period, followed by a four-day pathogen conidia exposure treatment period of the generalist entomopathogenic fungus *Metarhizium anisopliae*. Using these observations, we generated interaction networks and calculated a range of network level metrics and compared connectivity (degree centralisation, density and diameter) and structure (modularity and assortativity) between control and pathogen exposure treatments. We found that colonies of *C. fulvopilosus* responded to pathogen exposure by decreasing the number of connections (decreasing degree centralisation and density) whilst maintaining overall network connection (diameter), potentially limiting avenues for pathogen transmission. Colonies showed prophylactic modularity regardless of exposure and did not interact more with individuals sharing traits (assortativity). Thus, colonies of *C. fulvopilosus* appear to respond to pathogen exposure by limiting redundant interactions but maintaining colony connectivity for the transfer of resources and information.

Intended for submission to "Theoretical Ecology"

Introduction

Individual behaviours affect, and are affected by, the presence and behaviours of others within their social network. These social interactions therefore influence how information, resources and even pathogens are transmitted throughout the group (Makagon *et al.*, 2012). Interaction network analysis is an approach for examining and quantifying the associations that arise among interacting individuals; of particular interest are those interactions affecting the spread of pathogens (Eames & Keeling, 2002; Hamede *et al.*, 2009; Griffin & Nunn, 2012; Nunn *et al.*, 2015; Richardson & Gorochowski, 2015). Frequent interactions are expected to increase risks of pathogen transfer within networks (Otterstatter & Thomson, 2007), with larger networks expected to suffer from greater pathogen loads (Loehle, 1995; Altizer *et al.*, 2003). However, as group size increases there has been evidence for either, increases in pathogen loads (Nunn & Heymann, 2005) or decreases in pathogen loads (Rosengaus *et al.*, 1998). One potential factor which may mitigate the increased risk of spread associated with increased group size could be specific network structures, as the structure and dynamics of interaction networks are expected to impact pathogen spread (Keeling, 2005; Barthélemy *et al.*, 2008; Pei & Makse, 2013). Network analysis, based in mathematical graph theory, investigates relational properties between individuals, such as how unified a group or subgroup is, how interconnected individuals are, and individual positions within the network. A wealth of metrics describe social networks at multiple levels, from the level of the individual through to that of the network (see Makagon *et al.*, 2012 for review).

Assessing interaction networks in relation to pathogen transmission at the network level, we identified five network-level metrics which may play important roles in limiting pathogen spread across networks; degree centralisation, diameter, density, modularity and assortativity. Degree centralisation is calculated as the variation in degree centrality across an entire network, with higher scores suggesting that the network has many well-connected individuals. Degree centrality refers to how connected each individual is to others in the network. Individuals with a high degree centrality are at greater risk for infection as they interact with many partners (Christley *et al.*, 2005) and certain individuals act as "super-spreaders" due to their high degree (Lloyd-smith *et al.*, 2005). These individuals often act as bridges between groups and play an important role in managing network connectivity. Diameter represents the length of the shortest path required to traverse the entire network and has been shown to inhibit pathogen spread as it increases (Richardson & Gorochowski, 2015). Further, networks may share the same diameter but show very different levels of connectivity, as not all connections are required to

traverse a network, with certain connections being redundant, able to be removed without affecting diameter or overall network connection. Density refers to the proportion of all possible connections in a network that are realised and has been shown to drive pathogen spread rates (Otterstatter & Thomson, 2007), with infections spreading quicker in highly connected nests. Modularity is characterised as structures of social groupings, with collections of individuals experiencing greater within-group than between-group interactions (Newman & Girvan, 2004). Pathogen spread declines when interactions occur primarily within groups (Naug & Camazine, 2002) or in very modular networks. In a recent meta-analysis of group size and network measures related to pathogen transmission (Nunn et al. 2015 and references therein) it was found that modularity was related to increased network size. They showed that greater modularity increased the rate of disease transmission within groups but decreased disease transmission between groups, limiting the overall effects of pathogens in networks (Nunn *et al.*, 2015). Assortativity describes the extent to which interactions occur between individuals with shared traits (Newman, 2002). Increases in assortativity are expected to limit pathogen spread, in a similar manner as modularity, by decreasing between group interactions. These metrics are not independent of each other and depend on each other, therefore it is important to assess network structure using a range of metrics in order to develop a comprehensive and balanced understanding of interactions.

Eusocial insects represent an excellent model system for studying interaction networks due to their complexity and ease of maintenance. This complexity in eusocial societies arose largely through a division of labour and reproduction, which allows selection at both the individual and colony level (Waters & Fewell, 2012). Organisation in eusocial insects arises from the interplay between chemical communication and responses to dyadic interactions between nestmates (see Gordon, 2016; Leonhardt *et al.*, 2016 for reviews). In an assessment of colonies responding to starvation it has been shown that *Temnothorax albipennis* ants can alter network dynamics to facilitate the spread of food entering the nest (Sendova-Franks *et al.*, 2010), depicting a plastic interaction structure. As such ants represent an excellent model system for network analysis due to the interplay between individual based organisation and colony level selection, such as pathogen spread (Naug, 2008; Stroeymeyt *et al.*, 2014).

Of particular interest are behaviours, termed social immunity (SI; Cremer *et al.*, 2007), where social insects undertake behaviours at the individual level to limit pathogen risks at the colony level. These behaviours can be prophylactic such as showing assortativity between work groups

(Mersch *et al.*, 2013) or activated such as the self-removal of infected individuals (Chapuisat, 2010; Heinze & Walter, 2010). Social immunity interventions are expected to cause changes in network structure, either prophylactically or in response to pathogen intrusion. Eusocial insects have also been shown to identify and shift interactions in response to infections, by grooming infected nestmates more frequently than uninfected nestmates (Okuno *et al.*, 2012; Theis *et al.*, 2015) or by altering their behavioural patterns after interacting with infected cadavers of nestmates (Diez *et al.*, 2015). A growing area of research has focussed on variation in organisation by eusocial insect colonies to limit pathogen spread (Naug & Smith, 2007; Stroeymeyt *et al.*, 2014). How interaction networks shift in response to infection remains an important question, specifically, how do colonies exposed to a pathogen moderate network connectivity and structuring?

In this study, using experimental colonies of the eusocial ant *Camponotus fulvopilosus,* we assessed how colonies altered network connectivity and structure in response to pathogen exposure through video analysis. We generated interaction networks based on allogrooming and trophallaxis and quantified five network metrics: degree centralisation, diameter, density, modularity and assortativity at the network level. Networks, for colonies initially pathogen-free and later exposed to a pathogen, were constructed to compare shifts in network connectivity and structure. According to literature, we expected that density would decrease and diameter should increase. These changes should cause an overall decrease in connectivity in response to pathogen exposure, in order to limit avenues of spread. We expected that modularity and assortativity, representative of colony structuring, would increase to limit between group interactions facilitating the localization of infections within groups. Finally, we expect that degree centralisation would decrease to limit between-group interactions thereby also reducing the spread of pathogens.

Methods

Experimental setup

Three colonies of *C. fulvopilosus* were collected and maintained as per Chapter two. We utilised the generalist insect entomopathogen *Metarhizium anisopliae* (META69, isolate: ICIPE69) for pathogen exposures using Tween20 in line with studies of social immunity (Traniello *et al.*, 2002; Pie *et al.*, 2005; Okuno *et al.*, 2012; Tranter & Hughes, 2015; Qiu *et al.*, 2016). We used the methodology described in Chapter two for fungus cultivation, preparation and application. Preliminary assessments of mortality rates resulting from the exposure showed

that on average 83% of *C. fulvopilosus* maintained alone died, with only ~9% of these dying from causes other than *Metarhizium anisopliae* infections.

Experimental nests (±1300 mm^2 X 10 mm height, Figure 3.1 B) were established for each collected colony as per Chapter three, with colonies housed between two anti-reflective glass panels separated by JoinTech and attached to a foraging arena. Nests were kept in dark filming boxes and recorded under infrared illumination (Figure 3.2). Foraging arenas were exposed to a temperature (± 25 ºC) and humidity-controlled environment with a 12-hour day night cycle. Colonies were provided ad libitum access to sugar water and a protein source in the form of termites every three days. Each experimental nest included 100 randomly chosen worker ants, a queen and a mixture of brood, eggs and pupae from source colonies (±3 cm^2). Individuals were marked with numeric labels attached to their thorax using a clear nail polish as adhesive, following a procedure modified from Quevillon *et al.* (2015), labels were sufficiently small to limit effects on behaviour, movement or interactions. Queens were not labelled to reduce stress yet were visually distinct from nest-workers.

Experimental nests were allowed a 24-hour acclimation phase, during which the identity of any foragers (present in the arena) were noted. Forager checks were repeated three times daily for the duration of the trial to track the identity of any new foragers. Thereafter, 20% of foragers active in the foraging arena were collected, their identity noted, and sprayed, as per previous protocol (Chapter 1), with a 5% Tween 20 solution. Treated foragers were allowed to dry and were then returned to the foraging arena to reintegrate into the colony over 24-hours. Colonies were maintained for 4 days collecting ten-minute video recordings every two hours, representing the Tween treatment. On day five, a further 20% of active foragers in the foraging arena were collected, their identity noted, and they were sprayed, as per previous protocol (Chapter 1), with a 5% Tween solution containing 1 x 10^7 conidia of *M. anisopliae* per ml. Pathogen treated foragers were allowed to dry before returning them to the foraging arena to reintegrate into the colony over 24 hours. Thereafter, colonies were recorded for a further four days, for 10 minutes every two hours representing a fungal exposure treatment.

Forty-eight 10-minute behavioural observations per treatment were recorded for each colony. The duration and frequency of each allogrooming and trophallactic interaction was quantified for each behavioural recording. Interactions were only included if they persisted for more than three seconds. For each interaction the identity of participants was noted, trophallactic

interactions were characterised by mandible-to-mandible contact whilst allogrooming was characterised by the active grooming of one individual towards another.

Statistical Analysis

All analyses and visualisations were conducted in the R statistical environment (R-Core-Team, 2013), using the package "iGraph" (Csardi & Nepusz, 2015). From each observation, interaction networks were generated (available on request), with nodes characterised by functional group (queen, nest worker or forager) and forager status (untreated forager, Tween-treated forager or pathogen-exposed forager) and edges weighted by frequency of interactions. In order to visualise networks, we created networks based on the frequency of all interactions over the four days of Tween or pathogen treatment, with the layouts of contact networks generated based on the pathogen treatment data using the Fruchterman-Reingold algorithm (Fruchterman & Reingold, 1991), with attraction strength weighted by the frequency of interactions. For each interaction network we calculated, degree centralisation, density and diameter. Assortativity was assessed based on functional groups and forager status, which can range from -1 to 1, with negative values suggesting individuals interact with different status individuals and positive values suggesting they interact with individuals with the same status. Modularity was calculated using an implementation of the Walktrap community finding algorithm (Pons & Latapy, 2006). Modularity scores range from (-1, 1) with values closer to one suggesting greater structure compared to a random network. In order to determine how pathogen exposure affected interaction networks, we compared each metric per colony across treatment (Tween treatment or pathogen treatment) using Wilcoxon signed-rank tests with effect sizes following the classification of values smaller than 0.1 indicating a small effect, values between 0.1 and 0.3 indicating a moderate effect and values above 0.5 indicating a large effect.

Results

The broad scale interaction patterns for each *C. fulvopilosus* colony over the Tween treatment and the pathogen treatment were visualised using interaction networks weighted by the frequency of interactions (Figure 5.1). The number of interactions decreased in all three colonies after exposure to the pathogen, with some individuals breaking all connections within the nest. Queens did not interact with pathogen-treated individuals within any of the nests. Further, in colonies one and three, certain individuals that broke contact with the network represent pathogen treated individuals; however, this pattern did not hold for colony two.

All colonies of *C. fulvopilosus* responded to pathogen exposure by altering their overall number of interactions. Specifically two colonies decreased their density (i.e. proportion of interactions out of the maximum possible number of interactions) in response to pathogen exposure ($V = 956.5$, $Z = -3.77$, $p < 0.001$, $r = 0.84$, Figure 5.2 A Colony 1 ; $V = 668.5$, $Z = -2.02$, $p = 0.043$, $r = 0.45$, Figure 5.2 A Colony 3). These same two colonies reduced their reliance on well-connected individuals, namely degree centralisation, in response to pathogen exposure ($V = 809.5$, $Z = -2.27$, $p = 0.023$, $r = 0.51$, Figure 5.2 B Colony 1; $V = 721.5$, $Z = -2.30$, $p = 0.022$, $r = 0.51$, Figure 5.2 B Colony 3). However, one colony increased their density in response to exposure (increased, $V = 358$, $Z = -1.99$, $p = 0.047$, $r = 0.44$, Figure 5.2 A Colony 2). All colonies showed structuring with interactions primarily occurring within groups of individuals rather than between them, as shown by modularity scores close to 1 (Figure 5.2 C). Only colony one altered its degree of modularity in response to pathogen exposure by decreasing its structure ($V = 834$, $Z = -2.55$, $p = 0.011$, $r = 0.57$, Figure 5.2 C Colony 1). Colonies of *C. fulvopilosus* showed little consistency in structuring interactions based on functional group (queen, nest-worker or forager) and forager status (untreated forager, Tween-treated forager or pathogen exposed forager), with median assortativity values around zero and no changes occurring in response to pathogen exposure (Figure 5.2 D). Finally, network size remained consistent, neither increasing nor decreasing in response to pathogen exposure with no significant changes in network diameter occurring in any colonies (Figure 5.2 E).

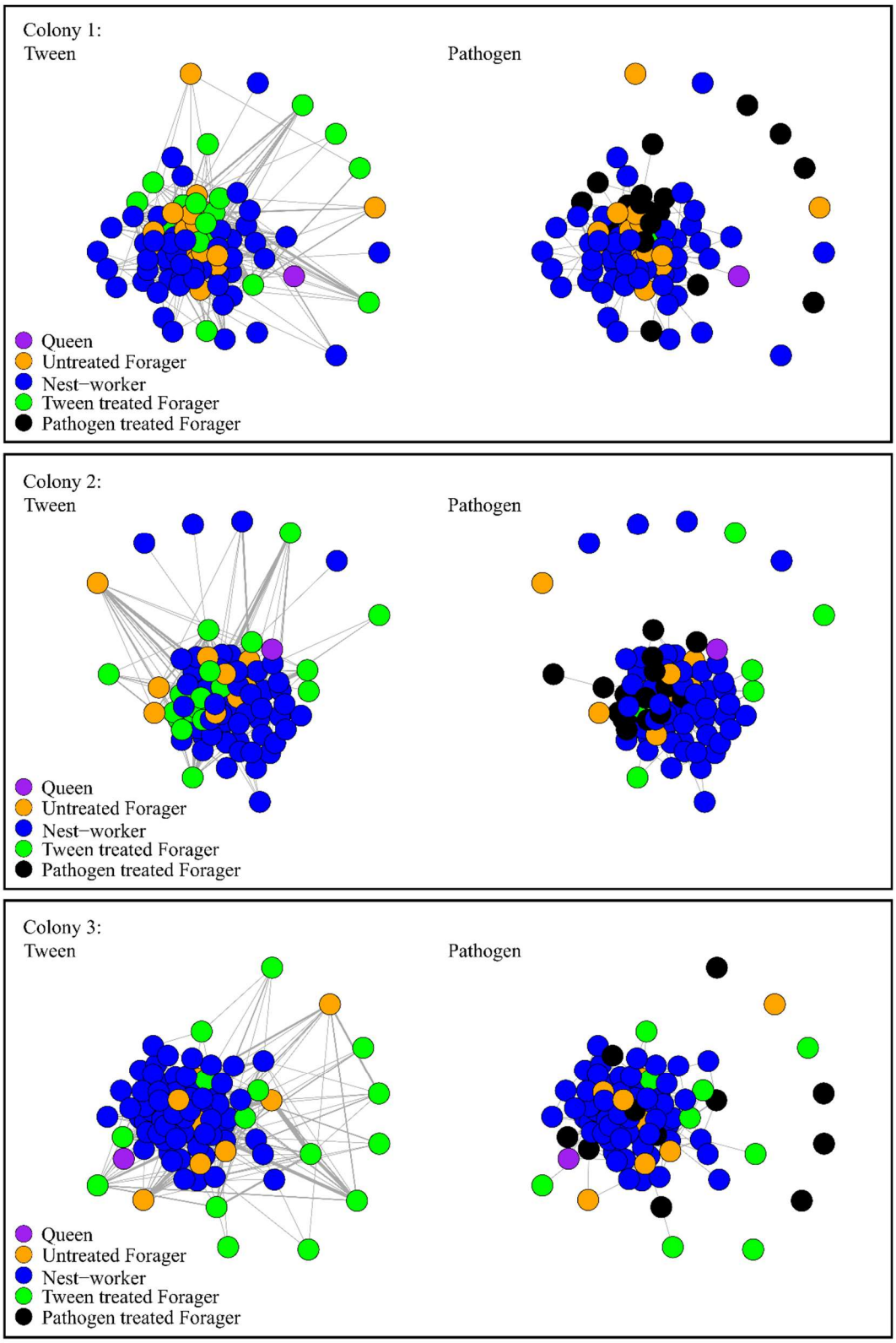

Figure 5.1: Visualisation of interaction networks for three colonies of *Camponotus fulvopilosus* under Tween treatment and after exposure to the entomopathogen *Metarhizium anisopliae.* Colours represent individual status (Purple: Queen, Orange: untreated Foragers, Blue: Nest-workers, Green: Tween-treated Foragers and Black: pathogen treated foragers) and width of connections represent the weighted connection between nodes based on the number of interactions. In cases where no Tween treated individuals occur in pathogen treatments, they were all exposed to the pathogen.

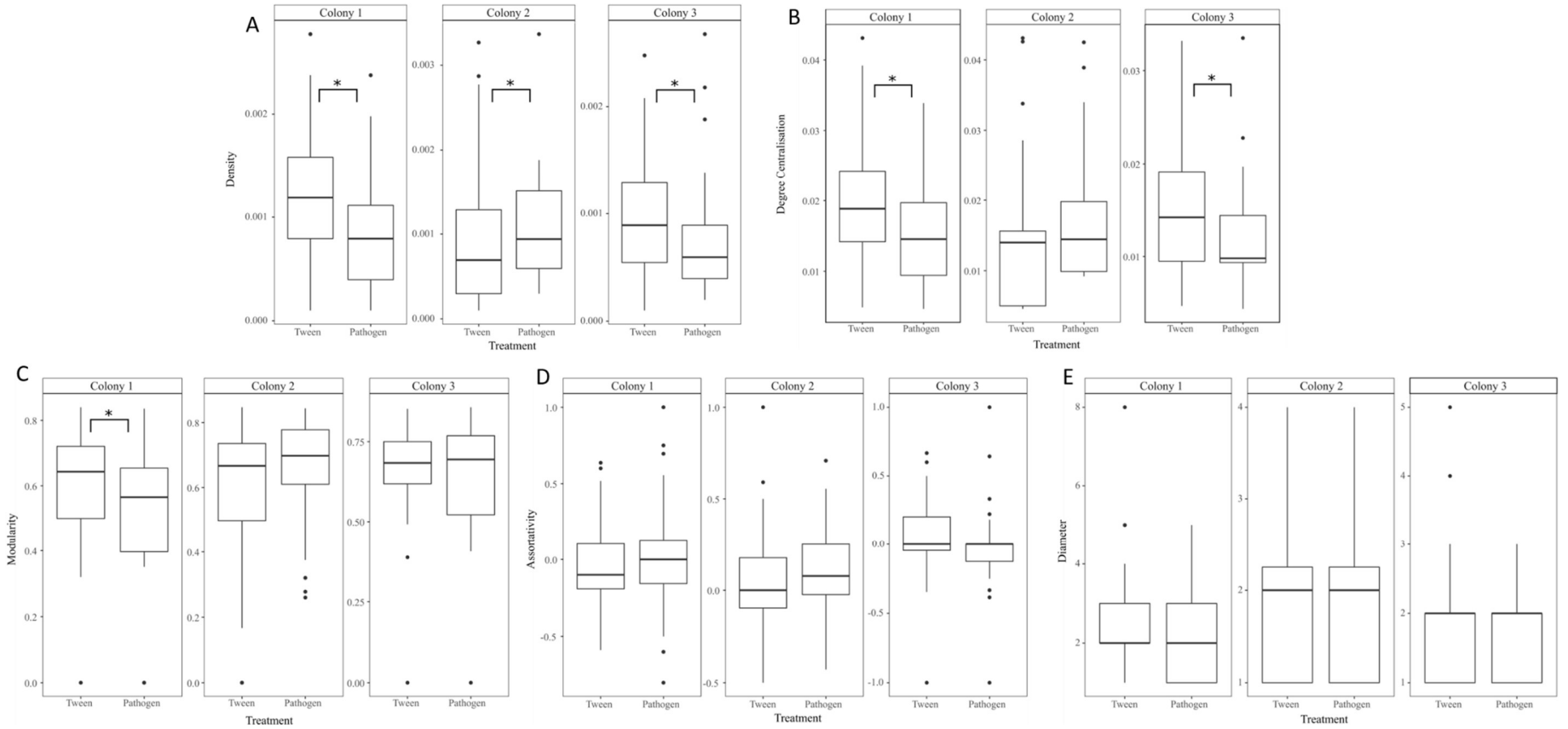

Figure 5.2: Boxplot depicting network shifts in A) Density, B) Degree centralisation, C) Modularity, D) Assortativity and E) Diameter across three colonies of *Camponotus fulvopilosus* in response to pathogen exposure. Solid lines depict medians, boxes represent interquartile range, whiskers represent min and max whilst dots represent outliers. * depict significant differences based on Wilcoxon sign-rank tests.

Discussion

Ants make an excellent model system for studying interaction networks due to their social complexity and ease of experimentation (Charbonneau *et al.*, 2013), particularly in relation to pathogen responses. Ants as eusocial organisms are expected to respond to infection or pathogen exposure to reduce pathogen spread rates at the colony level, despite potential individual level costs (Cremer *et al.*, 2007). Using colonies of the ant *C. fulvopilosus* as a model system, we found that colonies of *C. fulvopilosus* primarily responded to exposure by decreasing connectivity in the nest, through limiting interaction density and decreasing degree centralisation. Colonies engaged in fewer interactions in response to pathogen exposure and well-connected individuals were rarer. However, colonies did not alter the total network diameter in response to pathogen exposure, suggesting that overall network connectivity persisted whilst potentially limiting redundant interactions. Further, *C. fulvopilosus* did not alter network structure, with the degree of modularity or assortativity not shifting in response to pathogen exposure.

The primary mechanisms through which *C. fulvopilosus* responded to pathogen exposure was decreasing connectivity across the network. Colonies decreased both the density and their degree centralisation. Decreases in connectivity are expected to limit avenues for pathogen spread. Assessments of pathogen spread in *Bombus impatiens* showed that pathogen transmission was associated with network density, spreading to a larger degree in more connected colonies (Otterstatter & Thomson, 2007). Further, connections can be cut to protect important nest members such as the queen from pathogen encounter (Cremer *et al.*, 2007). Our findings match a recent study in *Lasius niger* where experimental colonies showed decreased density and degree centrality in response to treatment with an entomopathogenic fungi (Stroeymeyt *et al.*, 2018). Further they found that their decrease in degree centrality primarily occurred within foragers, which represent the individuals most likely to interact with pathogens (Cremer *et al.*, 2007). This decrease in connectivity may be facilitated by infected foragers withdrawing from the network by decreasing their interaction rates (Chapuisat, 2010; Heinze & Walter, 2010; Bos *et al.*, 2012). Shifts in interaction networks, together with SI behaviours, like self- and allogrooming (Reber *et al.*, 2011; Okuno *et al.*, 2012; Theis *et al.*, 2015) minimises pathogen load and drastically limit the impact of an infection at the colony level. Interestingly, the network diameter did not change, despite decreases in density and degree centralisation suggesting that while fewer interactions occurred under pathogen exposure, overall network connectivity persisted. A potential explanation for this is that redundant

connections were dropped in the pathogen treatment. This likely facilitates the continued spread of resources and information through the colony whilst decreasing superfluous connections which may facilitate pathogen spread (Stroeymeyt *et al.*, 2014). These responses to infection or pathogen exposure are expected to further limit the spread of disease when combined with modularity or assortativity.

Structured networks, with increased modularity and assortativity metrics, decrease pathogen spread rates by restricting infections to small frequently interacting groups (Naug & Camazine, 2002). Stroeymeyt and colleagues (2018) found consistent upregulation of modularity in response to infection in *Lasius niger*. Further, increased modularity in networks with low network structuring, limits pathogen spread to a greater degree than networks with high network structuring (Sah *et al.*, 2017). Moreover, increased modularity has been shown to increase the length of time a pathogen remains within a network (Salathe & Jones, 2010) and as such, colonies may not alter modularity in response to pathogen exposure or infection. Regardless of treatment, colonies of *C. fulvopilosus* maintained strong modularity, except for one colony that decreased modularity in response to pathogen exposure. It is likely that *C. fulvopilosus* does not rely on shifts in modularity to combat pathogen exposure but rather that network structuring already exists prophylactically, as has been detected in our work and previous studies (Mersch *et al.*, 2013; Quevillon *et al.*, 2015). One such prophylactic mechanism adopted by *C. fulvopilosus* for the control of infection might be the application of antimicrobials (Tragust *et al.*, 2013; Tranter & Hughes, 2015; Brütsch *et al.*, 2017). We found no clear pattern of assortativity, with individuals being as likely to interact with nestmates with similar traits as they are to interact with nestmates with dissimilar traits (but see Stroeymeyt *et al.*, 2018).

Our results, together with recent work on *C. pensylvanicus* (Quevillon *et al.*, 2015) and *Lasius niger* (Stroeymeyt *et al.*, 2018), support the idea that ant colonies can modify their interaction networks in response to pathogen exposure and infection and are a good model system for studying interaction networks (Charbonneau *et al.*, 2013). Future research should extend this body of knowledge by introducing more complexity such as nest structure, varying group sizes and incorporating multiple species. Studies have shown that nest structure plays an important role in the spread of disease (Pie *et al.*, 2004). The disease mitigating effects of spatial structure, interaction networks, and their synergism needs further exploration. Furthermore, in our study, experimental colony size was constrained by the limitations of manually tracking interactions.

The implementation of tracking algorithms (Mersch *et al.*, 2013; Crall *et al.*, 2018; Gernat *et al.*, 2018) can overcome these limitations, facilitating the expedient collection of data across larger colony sizes and a greater number of concurrent tracked individuals, assessing how networks of different sizes respond to infection. Further, interaction network analyses should be conducted on a wider range of species, as our work (see Chapters two and three) and other studies (Walker & Hughes, 2011; Tranter *et al.*, 2014) have shown that species respond and manage infections via a range of mechanisms. Finally, to fully understand how network structures affect pathogen dynamics, more studies should compare generated network patterning with randomised networks. This would facilitate evaluating whether a species' interaction network structure differs in a manner that may actually limit pathogen spread when compared to a null model and would provide valuable information for theoretical models of pathogen spread within colonies.

In summary, colonies of *C. fulvopilosus* decreased network connectivity in response to infection across the entire network with no consistent changes in modularity and assortativity. The network did not become more structured when faced with pathogen exposure but the number of interactions shifted in a manner to limit pathogen spread. *Camponotus fulvopilosus* was experimentally tractable and amenable to recording with interactions easily quantified. They also displayed interesting behaviours such as a reliance on chemical defences (Robertson & Zachariades, 1997), where we noted colonies engaging in what appears to be group level disinfection (see Figure S 3.1). As such, they represent a good model organism for interaction studies.

Chapter 5: Research contributions

DJP, with aid from TCW and CH, conceived and designed the experimental setup and analysis. DJP implemented the experimental setup with aid from Chris Du Toit in collecting and annotating observations. DJP processed and analysed the data. DJP wrote the paper with input from TCW and CH.

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Chapter 6: When to groom oneself or nestmates: Using matrix projection models to assess conidia removal in three South African ant genera.

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Abstract:

Ants likely contend with a range of pathogens including entomopathogenic fungi. High relatedness and frequent interactions place colonies at further risk. Ants combat this by exploiting various social immune mechanisms. Theoretically, ants rely on self-grooming and allogrooming to remove conidia within the first 48 hours of exposure, before conidia can germinate and cause infection. Reliance on these behaviours is expected to differ across ant species, based on their ecology and life history. Using empirical data we generated matrix projection models to assess conidia removal rates between three South African ant species, *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre*. Through knockout experiments we assessed the importance of self-grooming and allogrooming in removing conidia within 96 hours. Finally, we assessed how pathogen load affected conidia removal rates across species by increasing the number of initially infected ants and limiting exposure to foragers or exposing individuals across functional groups. All three species successfully removed all conidia within 96 hours of pathogen exposure. *Anoplolepis custodiens* relied primarily on allogrooming and *T. sericeiventre* relied primarily on self-grooming but benefitted from allogrooming, whilst *C. fulvopilosus* relied only on self-grooming. Conidia removal time increased with pathogen load. Interestingly we showed that in *A. custodiens* and *T. sericeiventre,* pathogen exposure limited to foragers took longer to manage than pathogenic conidia distributed throughout the colony. Thus, we show that all three species of South African ants were able to mitigate exposure to a generalist fungal pathogen; yet, each utilised different approaches to achieve this.

Intended for submission to "Journal of Theoretical Biology"

Introduction:

Social insects and ants in particular are characterised by dense colonies of related individuals, working together under closed, structured nest conditions (Hölldobler & Wilson, 1990). These conditions place colonies at risk of parasite and pathogen epidemics (Hamilton, 1987; Schmid-Hempel, 1995, 1998). The majority of ant species are characterised by an omnivorous and soilborne lifestyle, which predisposes them to infection by fungi, nematodes and helminths (Boomsma *et al.*, 2005). Foragers and waste workers are expected to be at greatest risk to fungal infection as they engage in dangerous tasks which may put them in contact with fungal conidia or ascospores (Cremer *et al.*, 2007). In particular they are at risk to infection by entomopathogenic fungi like *Metarhizium anisopliae* or *Beauveria bassiana* which propagate via cadavers and occur frequently in soil (Hajek and Leger 1994, Meyling and Eilenberg 2007 and references within). After conidia encounter a host, they adhere to the host cuticle, grow into the host and develop, killing the host via the compounds released and damage caused by fungal growth, thereafter the fungus grows out of the corpse and sporulates, releasing new infectious agents (Castrillo *et al.*, 2005). Entomopathogens are extremely effective and prevalent (Hughes *et al.*, 2004b; Reber & Chapuisat, 2012); however, conidia adherence within the first 48 hours is initially low, before the conidia has germinated and penetrate the hosts' cuticle (Vestergaard *et al.*, 1999). This provides hosts an opportunity to manage infections.

Despite the predicted high risk of infection, large scale epidemics are rare in social insects like ants (Evans, 1974). This has been attributed to their use of individual behaviours undertaken at potential energetic or health costs to themselves for the collective benefit of the colony and has been termed social immunity (SI; Cremer *et al.*, 2007). Social immunity behaviours are many and varied but of importance for preventing fungal infection is self and allogrooming. Self-grooming allows individuals to decrease their own cuticular pathogen load while allogrooming decreases the cuticular pathogen load of nestmates during the period of low conidia adherence (Reber *et al.*, 2011; Okuno *et al.*, 2012; Theis *et al.*, 2015). Studies on the relationship between allogrooming and pathogen encounter have produced varying outcomes, from no effect (Graystock & Hughes, 2011; Reber *et al.*, 2011; Theis *et al.*, 2015) to increased allogrooming rates (Hughes *et al.*, 2002; Walker & Hughes, 2009; Bos *et al.*, 2012; Okuno *et al.*, 2012) when exposed to infection. Furthermore, allogrooming can result in the secondary transfer of fungal conidia, as has been demonstrated in the ant *Lasius neglectus* (Konrad *et al.*, 2012), although in that case it did not result in subsequent mortality but rather provided increased resistance to subsequent infections. Further, multispecies studies have shown that there is substantial

variation in investment in self and allogrooming (Walker & Hughes, 2011; Tranter *et al.*, 2014) suggesting species make use of a range of SI behaviours to control pathogens.

Social insects have also been shown to utilise colony organisation to manage infections (Naug & Smith, 2007; Stroeymeyt *et al.*, 2014). This organisation acts through variation in spatial use (Baracchi & Cini, 2014; Quevillon *et al.*, 2015) and interaction heterogeneities (Mersch *et al.*, 2013) between workers engaged in different tasks and allows social insects to limit the impact of epidemics in their nests. *Lasius niger* foragers, who are expected to encounter more pathogens, utilised less nest space and interacted less with nestmates after infection (Stroeymeyt *et al.*, 2018). Whilst various SI behaviours are largely effective in mitigating the impacts of pathogen exposure, these interventions are not absolute. Studies exploring the limitations of SI primarily explore them through dose responses by altering the concentration of pathogen exposure to single individuals (Reber *et al.*, 2011; Pull *et al.*, 2018). Very few studies have assessed how the number of infected individuals, as well as the task-associated identity of the infected individuals, affect SI interventions (but see, Jaccoud *et al.*, 1999). Identifying these potential limitations to SI requires substantial experimental work, alternatively with mathematical models, in conjunction with experimental data, this process may be fast-tracked.

The spread of disease can be modelled using deterministic (rules-based method) or stochastic (allowing natural variation in rule implementation) processes. Furthermore, models can be implemented in many different manners i.e. mass action models, cellular automata, agent-based models or matrix projection models, depending on the scale at which calculations are undertaken. A multitude of theoretical epidemiological studies have been conducted with social insects as the subject (Naug & Camazine, 2002; Pie *et al.*, 2004; Fefferman & Ng, 2007; Fefferman *et al.*, 2007; Naug, 2008; Wilson-Rich *et al.*, 2009; Hock & Fefferman, 2012; Konrad *et al.*, 2012; Novak & Cremer, 2015). These studies have assessed the spread of disease in social insects by including a range of disease prevention strategies.

Naug and Camazine (2002) created a cellular automaton, with which they assessed how division of labour, interaction networks and colony demography affected disease spread within a colony. They found that alone, these factors could not limit disease spread; however, in combination they limited the epidemic (Naug & Camazine, 2002). Using both a cellular automaton and a mean field approximation method, Pie and colleagues (2004) assessed the effects of worker density, activity level, probability of disease transfer and nest structure on

epidemics. They found that disease transfer rate and the population density acted synergistically in enhancing epidemics, whilst decreased worker movement and increased nest structure slowed disease spread (Pie *et al.*, 2004). Fefferman et al. (2007) created a series of cellular automata to compare the relative effectiveness of mechanisms, such as: immunity through immune priming, nest hygiene, allogrooming, colony age, colony demographics and nest arrangement. Nest hygiene presented the most effective control method assessed although immunity remained an effective control mechanism. The effectiveness of allogrooming varied depending on disease conditions, increasing epidemics when pathogen exposure was periodic, but decreasing epidemics when pathogen exposure was continuous. They found that homogeneity in age facilitated disease spread and that spatial arrangements of workers had little effect on epidemics (Fefferman *et al.*, 2007). Konrad et al. (2012) created a Susceptible-Infectious- Removed-iMmune (SIRM) model using ordinary differential equations to assess social immunisation effects on epidemics. They compared active immunisation (immune priming), where immunity is developed as a result of infection, to passive immunisation, where immunity is passed on from an infectious member. Passive immunisation led to a larger proportion of immune individuals; however, active immunisation lowered death rates and eliminated the disease sooner (Konrad *et al.*, 2012). More recently Theis et al. (2015) created small scale stochastic Susceptible-Infected-Susceptible (SIS) models based on empirically collected data, to feed into a larger scale deterministic model assessing self and allogrooming effects on epidemics. Allogrooming between healthy individuals and infected individuals was potentially beneficial or harmful to the healthy individual depending on the modelled efficiency of allogrooming and the pathogenicity of the disease (Theis *et al.*, 2015), supporting findings from previous epidemic models (Fefferman *et al.*, 2007).

Whilst the field of insect epidemiology is growing, there remain gaps in model execution, with most studies applying general infection dynamics as opposed to implementing biologically relevant dynamics related to their primary pathogens. The majority of studies assessing epidemics empirically in social insects, particularly ants and termites, make use of obligate killing entomopathogenic fungi, such as *Metarhizium anisopliae* or *Beauveria bassiana.* These fungi adhere to the host's cuticle before germinating and infecting the host and can thus be removed or transferred through interaction, before infection occurs. In a recent study a deterministic model was applied that implicitly accounted for the life history of entomopathogenic fungi. It was found that even increased contact rates without any social immunity aspects, such as allogrooming or conidia deactivation, can dilute pathogen loads

below disease causing levels through the simple transfer of conidia via any physical contact (Novak & Cremer, 2015). To our knowledge, no study has assessed specifically the window of opportunity directly after fungal infection (Vestergaard *et al.*, 1999) during which grooming can mitigate any infection risk by removing conidia from exposed individuals.

Therefore, this study explicitly explores the short window of opportunity for conidia removal by using experimental data (Chapters 2 and 3) from three South African ant species: *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre*, to build matrix projection models. Using these models, we compare the efficacy of the three species in mitigating infection risks under standard conditions. Thereafter we theoretically knock out allogrooming or self-grooming to assess the importance of these mechanisms in each species. Finally, we compare different infection regimes infecting more members of the colony: low and medium dose restricted to foragers as well as a medium and high dose across the colony. We expect that *A. custodiens* will mitigate infections in the shortest time frame, followed by *T. sericeiventre* and then *C. fulvopilosus*. These expectations are based on our previous findings (see Chapter 2 and 3), where *A. custodiens* showed the greatest allogrooming frequency, followed by *T sericeiventre* and then *C. fulvopilosus,* which showed inefficient allogrooming. Further, we expect that conidia removal rates will be greatest when models include a combination of both allogrooming and self-grooming. We expect the lowest conidia removal rates in allogrooming only models, while models incorporating self-grooming will show intermediate conidia removal (see Chapter 2) in line with our empirical data that indicated self-grooming was more effective than allogrooming in removing conidia. We expect that as more individuals are initially exposed the time taken to remove conidia will increase and that removal rates will be greater when only foragers are exposed to pathogens as ants have likely developed adaptations to mitigate exposure in foragers given their increased likelihood to encounter pathogens (Boomsma *et al.*, 2005; Ugelvig & Cremer, 2007).

Methods:

In order to assess how three species of South African ants manage exposure to entomopathogenic fungi we generated matrix projection models. These models were built using the data collected in previous work to simulate the effect of grooming (Chapter 2) and interaction rates (Chapter 3) on conidia removal during the period of low conidia adherence (Vestergaard *et al.*, 1999). Our matrix projection model followed the basic structure of:

$$\text{Equation 1: } N_{t+1} = A_t N_t$$

where N_t represents a vector comprised of conidia loads on 100 individuals at time t, and A_t represents a transmission matrix at time t, with the element a_{ij} on the i-th row and j-th column of A_t representing the probability of a conidia being transmitted from individual j to i during one time unit. Transmission matrices were generated per time step, representing one hour, using data collected in previous chapters. Using matrix multiplication, we determined conidia loads at time t+1 as the product of the transmission matrix and the vector of conidia loads at time t.

Standard model simulations ran as follows. Models were initialised by assigning each individual to a functional group: foragers (20), nest workers (79) and queen (1), after which an initial exposure was implemented. In the standard model design, 10 foragers were randomly assigned a conidia load of 1000. Thereafter, for each time step, a transmission matrix was generated. This was accomplished by assessing all possible pairwise interactions, as stochastic events, based on the functional type of interacting individuals using observed interaction rates to estimate the probability of encounter during each time step and the effect of conidia removal and transmission based on observed allo/self-grooming effects and secondary transmission rate (Data from Chapter 2 and 3, Table 6.1). More specifically, the transmission matrix was generated via a two-step process where we first determined whether interactions occurred, based on observed interaction rates (see Chapter 3). These interaction rates were calculated from empirical data. They were calculated as the mean proportion of interactions that occurred between two functional types out of the total possible number of interactions, furthermore interactions with oneself were guaranteed. Thereafter, wherever interactions occurred, we estimated the transmission rate based on observed effects of grooming and secondary transmission rates (see Chapter 2, Table 6.1) using the following equations:

$$\text{Equation 2: } a_{ij} = e_{ij}t_{ij} - e_{ji}t_{ji};\ \text{if } i \neq j$$

$$\text{Equation 3: } a_{ii} = 1 - e_{ii}s_i - \sum_{j \neq i} e_{ij}a_{ij}$$

where e_{ij} represents the rate of directional interaction from j to i (presumably observed allogrooming probability of j on i during one time step), with $e_{ii} = 1$ (guaranteed self-grooming); t_{ij} is the secondary transfer rate of directional interaction from j to i (i.e. the rate of individual i receiving conidia from the allogrooming actions from j to i); t_{ji} is the secondary transfer rate from i to j with individual i as the donor of allogrooming; s_i the effect of self-grooming and a_{ij} the effect of allogrooming. We normalised a column of transmission matrices to 1 if the sum of this column is greater than 1 (to ensure the number of conidia contributed from one individual to others cannot exceed the number of conidia it carries in the previous step, noting the stochastic process of assigning matrix elements), and we multiplied by the vector of conidia loads from the previous timestep to generate the new conidia load. This process was repeated 96 times representing 4 x 24-hour days, as conidia are expected to germinate and penetrate hosts within 24-48 hours (Vestergaard *et al.*, 1999) and this allowed for twice that time window. Total conidia load at each time step was recorded and total time taken to remove all conidia from the nest was calculated or set to 96 in cases where conidia still remained at the end of a run.

In order to compare species, the general model procedure was implemented for each species using the previously collected data (Table 6.1), and 1000 simulations were run. In order to assess the importance of self and allogrooming we generated knockout models where transmission rates for self-grooming or allogrooming were set to zero and simulated 1000 times each. Finally, in order to assess how infection load affected conidia removal rates we altered the initial infection to represent a low, medium, medium dispersed and high dispersed dose. A low dose followed the standard procedure infecting 10 foragers, the medium dose infected all 20 foragers. The medium dispersed dose infected 20 individuals across the colony except for the queen and the high dispersed dose infected 40 individuals except the queen across the colony. We ran 1000 simulations of each treatment for each species.

Statistical analysis:

Models were created and run in Mathematica version 12.0 (Wolfram Research, 2019) and all statistical analyses were conducted using R (R-Core-Team, 2013). We compared: i) rates of conidia removal (the time taken to remove all conidia in simulations) across species; ii) the effectiveness of self-grooming and allogrooming on conidia removal rates (standard models, no self-grooming models and no allogrooming) for each species and iii) conidia removal rates across different doses with Kruskal-Wallis tests followed by Dunns tests using the Benjamini-Hochberg corrections for post hoc analysis. Further, for comparison we calculated mean and standard deviations in total conidia load over time for all treatments to visualise simulations.

Table 6.1: Interaction rates, transmission rates and model specifications for standard modelling procedure as well as the initiation specifications.

Value	Definition	*Anoplolepis custodiens*		*Camponotus fulvopilosus*		*Tetramorium sericeiventre*	
Interaction likelihood used in determining e_{ij}							
FF	Forager-Forager	0.0928		0.0383		0.125	
FQ	Forager-Queen	0		0		0	
FW	Forager- Worker	0.0181		0.00684		0.0397	
QF	Queen-Forager	0		0		0	
QW	Queen- Worker	0.00775		0		0.0278	
WF	Worker-Forager	0.0987		0.0112		0.0820	
WQ	Worker-Queen	0.491		0		0.194	
WW	Worker-Worker	0.0282		0.00328		0.0267	
Transmission rate		**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
S	Self-grooming	0.191	0.00616	0.241	0.0237	0.281	0.0143
G	Allogrooming	0.102	0.0303	0.00862	0.00557	0.0167	0.0118
T	Secondary transfer	0.00205	0.00658	0.0135	0.0666	0.00738	0.0234
Initiation specifications		Starting conditions		Infection conditions			
				Standard	Medium	Medium Spread	High
		Queen	1	0	0	0	0
		Worker	79	0	0	20	40
		Forager	20	10	20		

Results

The three species differed significantly in how quickly they removed conidia present in their colonies during simulations (KW: $H_2 = 2751.7$, $p < 0.001$, Figure 6.1, all post hoc tests differed significantly). *Anoplolepis custodiens* overcame exposure the fastest followed by *T. sericeiventre* and then *C. fulvopilosus*, with all species able to remove all conidia within 48 hours (Figure 6.1).

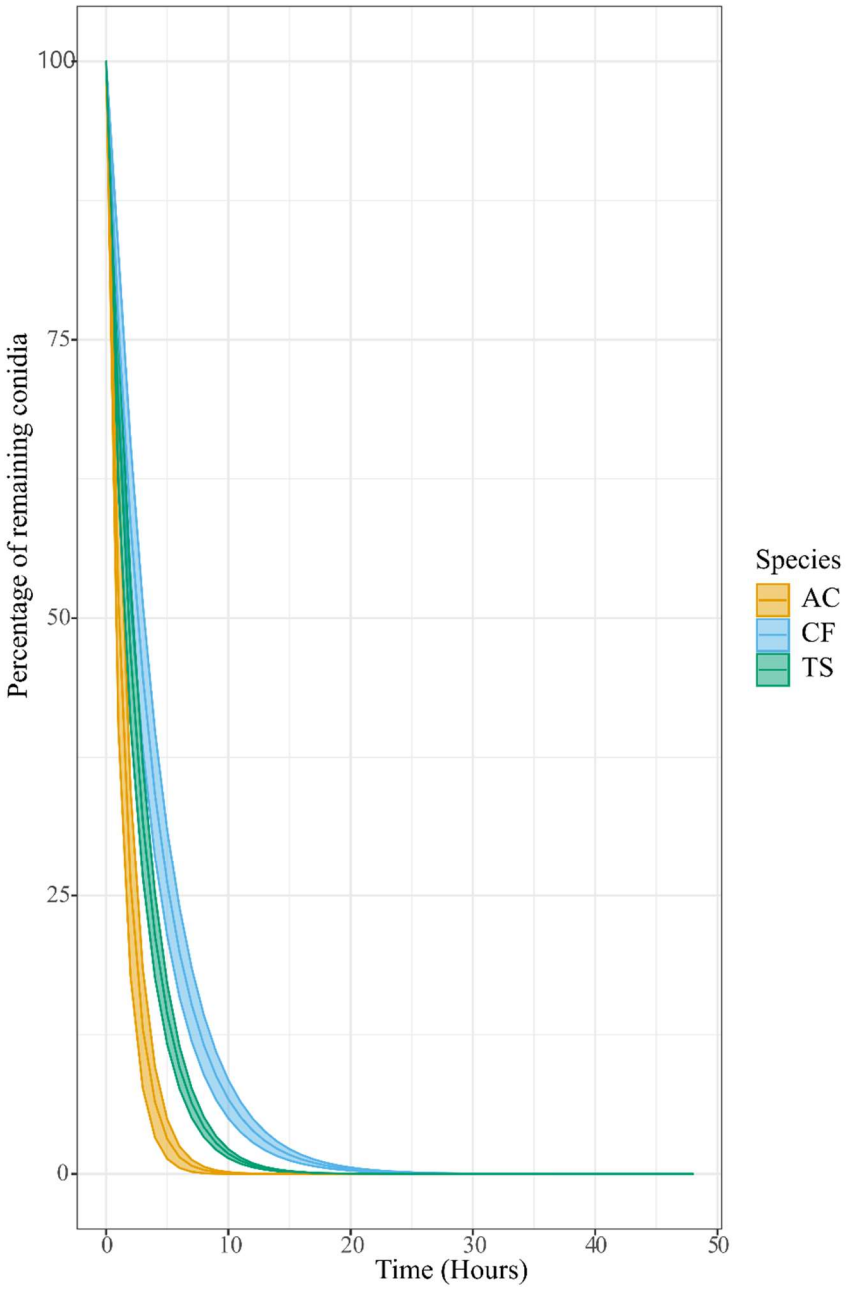

Figure 6.1: Mean and 95% CI of percentage of initial pathogen conidia load remaining in colonies over time in hours. Colours represent species identity with *Anoplolepis custodiens* represented in orange, *Camponotus fulvopilosus* in blue and *Tetramorium sericeiventre* in green. Means and 95% CI were calculated based on 1000 simulations.

Behavioural knockouts affected conidia removal rates in *A. custodiens* (KW: $H_2 = 2721.1$, $p < 0.001$, Figure 6.2 A, all post hoc tests significantly different). Allogrooming had the largest effect on conidia removal, since knocking out allogrooming lead to extended time for conidia to be removed. Colonies without self-grooming also took longer to remove conidia than the standard conditions (Figure 6.2 A).

Anoplolepis custodiens were able to remove all the conidia, irrespective of dose, well within the allotted 96-hour time frame. However, the time taken to remove all conidia were significantly different across the simulated doses (KW: $H_3 = 1865.2$ $p < 0.001$, Figure 6.2 B all post hoc tests differed significantly). Conidia were removed quickest in the medium dispersed dose followed by the high dispersed dose, thereafter the standard dose was quickest, and the medium forager- restricted dose took the longest to be dealt with (Figure 6.2 B).

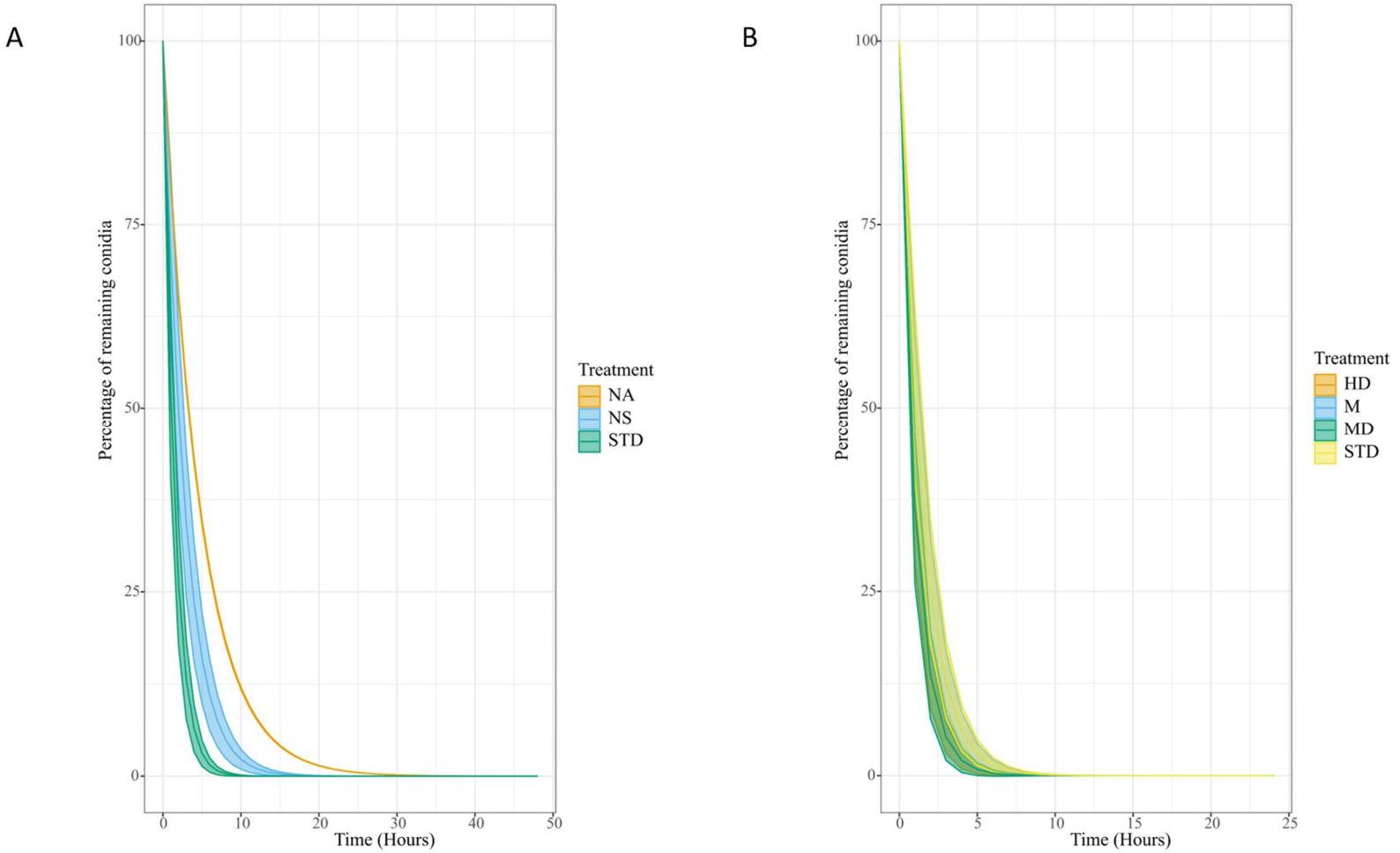

Figure 6.2: Mean and 95% CI of percentage of initial pathogen conidia load remaining in colonies of *Anoplolepis custodiens* over time in hours. A: represents behavioural modifications, colours represent treatment with standard conditions (self- and allo-grooming) represented in green, no allogrooming in orange and no self-grooming in blue. B: depicts dose responses with yellow representing low dose conditions, orange representing a high dispersed dose, blue a medium dose and green a medium dispersed dose over the colony. Due to substantial overlap in 95% CIs the distinction between treatments is not apparent in B. Means and 95% CI were calculated based on 1000 simulations.

Behavioural modifications affected the speed of conidia removal during simulations for *C. fulvopilosus* (KW: $H_2 = 2272.8$, $p < 0.001$, all post hoc tests differed significantly, Figure 6.3 A). Simulations where ants did not have access to allogrooming generated the quickest conidia removals, followed extremely closely by simulations with access to both self-grooming and allogrooming (Figure 6.3 A). Despite significant differences occurring between the two treatments, the average differences were less than a single timestep. Ants not having access to self-grooming, were unable to remove all conidia within the time frame but managed to decrease conidia by on average 96% (Figure 6.3 A). *Camponotus fulvopilosus* responded differently to all dose simulations (KW: $H_3 = 3238.3$, $p < 0.001$, all post hoc tests differed significantly, Figure 6.3 B). Ants removed all conidia in simulations with low doses in the shortest time period followed by the medium dispersed dose, medium dose and finally the high dispersed dose (Figure 6.3 B).

Behavioural knockouts in simulations of *T. sericeiventre* resulted in differences in the time taken to remove all the conidia across the treatments (KW: $H_2 = 2837.4$, $p < 0.001$, all post hoc tests differed significantly, Figure 6.4 A). Conidia removal occurred quickest in colonies with access to both self-grooming and allogrooming, followed by colonies with no allogrooming. Colonies without self-grooming took the longest to remove conidia and in some of the simulations, were unable to remove all conidia within the allotted 96-hour timeframe (Figure 6.4 A). Simulated doses greatly affected the rate of conidia removal in *T. sericeiventre* with dosage differing significantly in most cases (KW: $H_3 = 2864$, $p < 0.001$, Figure 6.4 B). Conidia removal occurred fastest in the medium dispersed dose followed by the high-dispersed dose and standard dose which did not differ significantly, with ants taking the longest to remove all conidia in the forager-restricted medium dose (Figure 6.4 B).

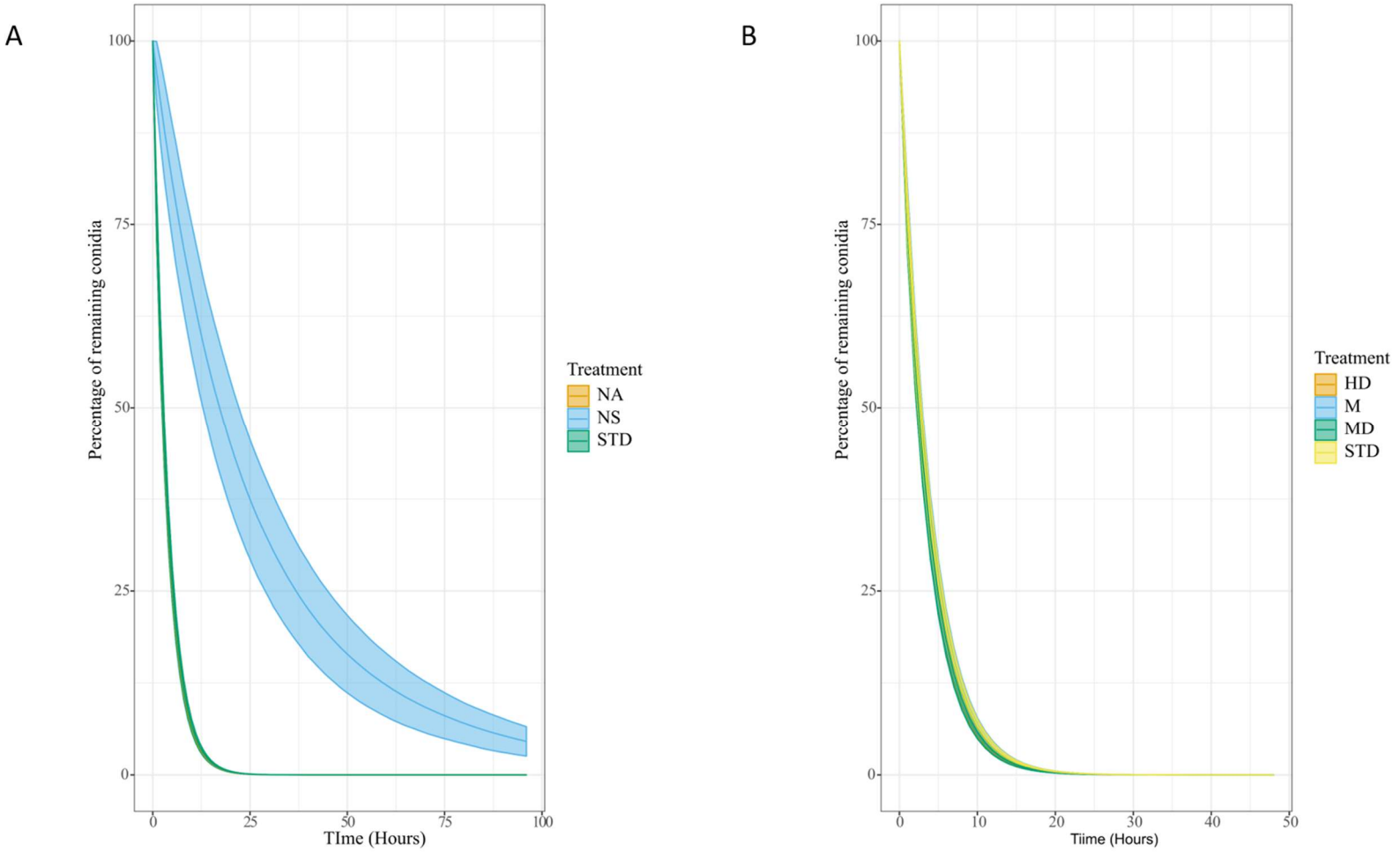

Figure 6.3: Mean and 95% CI of percentage of initial pathogen conidia load remaining in colonies of *Camponotus fulvopilosus* over time in hours. A: represents behavioural modifications, colours represent treatment with standard conditions (self- and allo-grooming) represented in green, no allogrooming in orange and no self-grooming in blue. B: depicts dose responses with yellow representing low dose conditions, orange representing a high dispersed dose, blue a medium dose and green a medium dispersed dose over the colony. Due to substantial overlap in 95% CIs the distinction between treatments is not apparent in A and B. Means and 95% CI were calculated based on 1000 simulations.

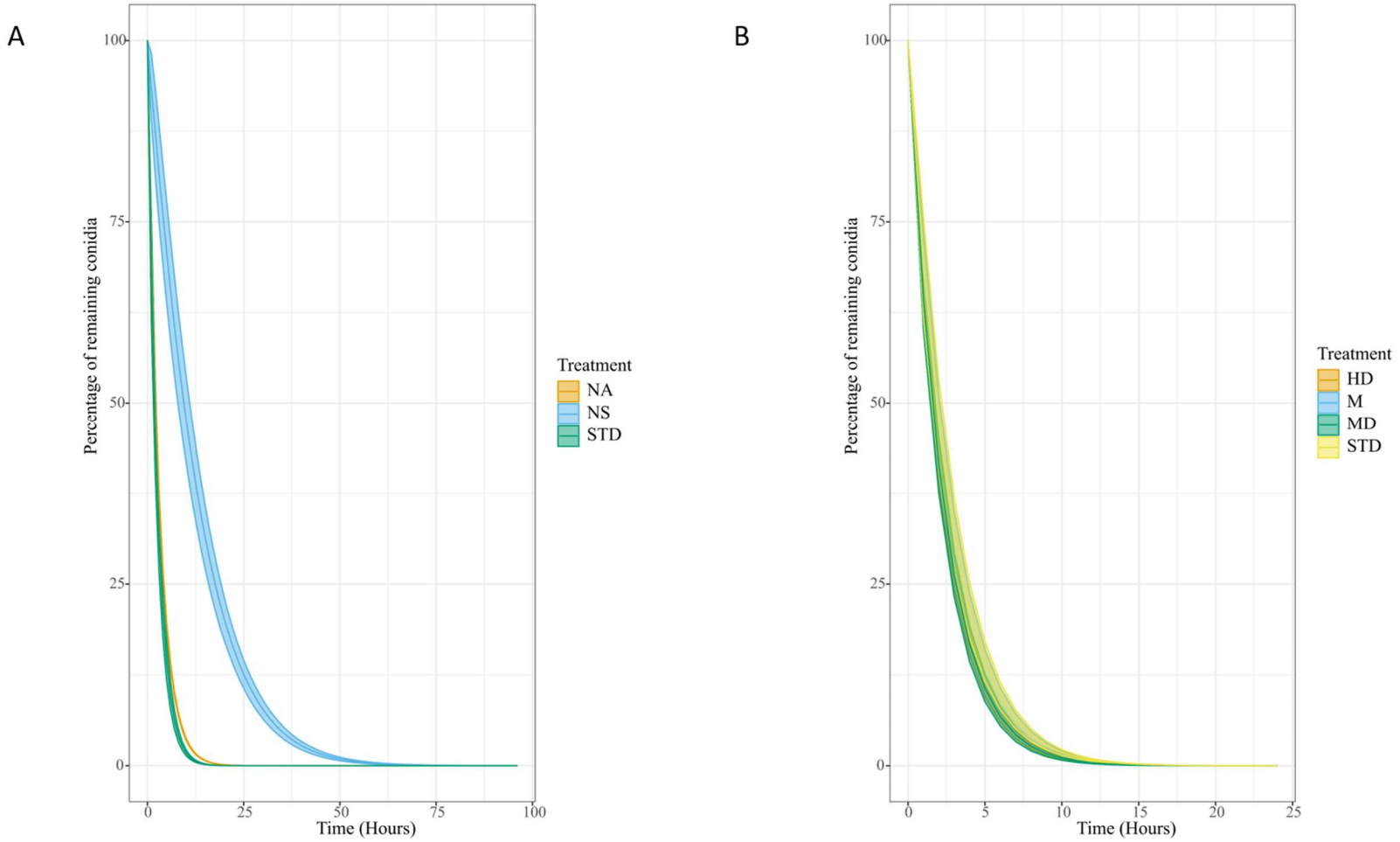

Figure 6.4: Mean and 95% CI of percentage of initial pathogen conidia load remaining in colonies of *Tetramorium sericeiventre* over time in hours. A: represents behavioural modifications, colours represent treatment with standard conditions (self- and allo-grooming) represented in green, no allogrooming in orange and no self-grooming in blue. B: depicts dose responses with yellow representing low dose conditions, orange representing a high dispersed dose, blue a medium dose and green a medium dispersed dose over the colony. Due to substantial overlap in 95% CIs the distinction between treatments is not apparent in B. Means and 95% CI were calculated based on 1000 simulations.

Discussion

All three ant species were able to completely eradicate all conidia within a 48-hour window in simulations of an entomopathogenic fungus exposure. As such, all three species should successfully withstand a low exposure to entomopathogenic fungi such as *Metarhizium anisopliae* or *Beauveria bassiana*. These pathogens occur readily near ant nests and are prevalent in their local environments (Keller *et al.*, 2003; Hughes *et al.*, 2004b; de Zarzuela *et al.*, 2012; Reber & Chapuisat, 2012). These pathogens must infect hosts with sufficient conidia to overcome their immunity, with ants able to survive low doses (Hughes *et al.*, 2004a). Our simulations suggest that there is variation in the primary mechanism for conidia control across the three species, self-grooming was the primary mechanism in *C. fulvopilosus* and *T. sericeiventre;* but, allogrooming was the primary mechanism for *A. custodiens.* Although self-grooming contributed to conidia removal in all three species, allogrooming was unimportant in *C. fulvopilosus*. Higher doses did take longer to manage in our simulations, although if infections began with nest workers, they were dealt with quicker in *A. custodiens* and *T. sericeiventre* than in infections restricted to foragers.

We expected that *A. custodiens* would deal with conidia loads the quickest, of the three species assessed, given their high frequency of allogrooming. Our findings supported this, with *A. custodiens* performing the best at removing conidia in simulations. *Anoplolepis custodiens*, is characterised by extremely high activity levels (Addison & Samways, 2006) and from our previous work we have shown very high interaction rates for this species (see Chapter 3). We presume that their high rates of interaction and effective allogrooming removed all the conidia in a timely manner despite their low (in relation to *C. fulvopilosus* and *T. sericeiventre*) self-grooming rates. Interestingly, *A. custodiens* relied on allogrooming as their primary mechanism for conidia control as opposed to self-grooming. But previous work has shown that *Lasius japonicus* upregulated allogrooming over self-grooming in response to pathogen exposure (Okuno *et al.*, 2012). Similarly, in the red imported fire ant allogrooming was an important aspect of managing pathogen exposure playing a more prominent role than self-grooming (Qiu *et al.*, 2014), as in our work access to both self and allogrooming results in the greatest conidia removal.

Camponotus fulvopilosus showed an inefficient allogrooming capacity (see Chapter 2) and our predictions that they would perform the worst in our model simulations were confirmed. Yet, despite performing the worst they were still able to remove all conidia within the 48 hours, before conidia are expected to germinate. The primary mechanism employed by *C. fulvopilosus*

was self-grooming and when this behaviour was removed from the model, *C. fulvopilosus* was unable to remove all conidia within the 96-hour timeframe. Allogrooming in *C. fulvopilosus* was expected to be less important than self-grooming given our previous findings (see Chapter 2). We did not expect it to have no discernible effect, with it's removal from the model barely affecting conidia removal rates compared to its inclusion in the model. The extremely low rate of interactions between nestmates of *C. fulvopilosus* (see Chapter 3) offer little opportunity for allogrooming to occur and with its low efficacy (see Chapter 2) may potentially explain this finding. Alternatively, it could represent a reliance on alternate mechanisms such as chemical control, as they are well known for their proclivity to spray formic acid as a defence mechanism (Robertson & Zachariades, 1997) or even a greater innate immunity through melanised cuticles (Feldhaar & Gross, 2008; José De Souza *et al.*, 2011; but see Sinotte *et al.*, 2018). Consequently, individuals exposed to conidia appear to deal with the conidia through self-grooming before there is a chance of the conidia germinating.

Tetramorium sericeiventre showed intermediate performance levels when compared to *A. custodiens* and *C. fulvopilosus,* as was expected from our previous work (see Chapter 2). *Tetramorium sericeiventre* shows similar interaction rates to those of *A. custodiens;* however, their allogrooming was less efficient than their self-grooming efficiency which was the highest of the three species. As such it is not unexpected that they would primarily rely on self-grooming. Without access to self-grooming they were, in some of the simulations, unable to remove conidia in the given period. However, unlike *C. fulvopilosus,* they still benefitted from allogrooming as our simulations showed. Studies have found that ants maintained in groups with access to both self-grooming and allogrooming are likely to have the lowest conidia loads (Qiu *et al.*, 2014). *Tetramorium sericeiventre* represent the smallest colonies numerically of the three species assessed and may not be able to rely as extensively on nestmates as *A. custodiens* does or chemical control as *C. fulvopilosus* potentially does. They may have to rely on multiple mechanisms (i.e. self-grooming and allogrooming) of pathogen control, invest in greater innate immunity or even implement novel mechanisms. A recent study has shown that small colonies of ants will implement alternate mechanisms in managing infection, such as evacuating nests in response to infection before opting to clean the nest (Leclerc & Detrain, 2018).

Our simulations identified self-grooming as the primary mechanism for conidia removal in two species, yet self-grooming was effective across all three species. Removing self-grooming from the model increased the time taken to successfully remove all the conidia. In the case of all *C. fulvopilosus* simulations and some *T. sericeiventre* simulations removing self-grooming prevented complete pathogen control within the 96-hour timeframe. Self-grooming has been shown to occur throughout the day when colony members are not actively involved in other tasks (Charbonneau *et al.*, 2013) and it is also increased in response to infection (Morelos-Juárez *et al.*, 2010). We propose the high reliance on self-grooming stems from it being an ancestral trait exploited by most insects (Hlavac, 1975). Potential costs for self-grooming are low as their benefits vastly outweigh their cost, with grooming decreasing an individual's likelihood of succumbing to infection. Self-grooming can also be facilitated with chemical control such as using acidopore and metapleural gland (Graystock & Hughes, 2011; Yek & Mueller, 2011; Tranter & Hughes, 2015; Tranter *et al.*, 2015) secretions to improve conidia inactivation. Our previous work (Chapter 2) has shown that self-grooming is important for limiting pathogen load and it is upregulated in response to infection (Reber *et al.*, 2011). Our simulations showed that colonies were less able to effectively manage infections without self-grooming, but allogrooming was important in two of the species assessed.

Allogrooming is expected to play an important role in mitigating pathogen risks by facilitating the removal of conidia (Walker & Hughes, 2009; Reber *et al.*, 2011). Further, ants have been shown to alter their allogrooming rates in response to infection, with infected individuals receiving more frequent allogrooming events in the initial two days following infection (Bos *et al.*, 2012). Allogrooming was the primary mechanism employed by *A. custodiens* for conidia removal and was effective in decreasing the time taken to remove conidia for *T. sericeiventre. Camponotus fulvopilosus* did not benefit substantially from the inclusion of allogrooming. Using cellular automata models, Fefferman and collegues (2007) found that allogrooming decreased survival under constant pathogen threat; but, when infections were periodic, allogrooming increased colony survival. Periodic infections are more likely in the case of generalist pathogens like *Metarhizium anisopliae,* whilst constant pathogen threats are more likely with specialist fungal pathogens like *Ophiocordyceps* (Loreto *et al.*, 2014). Allogrooming may provide a secondary benefit beyond conidia removal by increasing the number of individuals exposed to the pathogen potentially facilitating immune priming (Ugelvig & Cremer, 2007; Konrad *et al.*, 2012; see Masri & Cremer, 2014 for review). If colonies are exposed to a variety of pathogens and are relying on immune priming, they may

increase the risks of an epidemic developing. A recent study has shown that ants are at greater risk of succumbing to a novel pathogen if they are recovering from exposure to a previous pathogen (Konrad *et al.*, 2018). Although, they manage these risks through individuals altering their allogrooming rates in response to prior infections and the dose of the infection (Konrad *et al.*, 2018).

Camponotus fulvopilosus took longer to remove conidia as conidia doses increased, following our expectations. Quite interestingly, we found that in *A. custodiens* and *T. sericeiventre,* high and medium conidia doses affecting individuals across the colony were dealt with quicker than low and medium doses that were restricted to foragers. Although, regardless of who was exposed, simulations with a greater number of exposures took longer to manage. Most social insect colonies use age polyethism (Camargo *et al.*, 2007; Griffiths & Hughes, 2010) where older workers engage in risky behaviours such as foraging during which they are expected to encounter pathogens (Cremer *et al.*, 2007). Foragers are therefore more expendable and may manage their pathogen loads less through grooming and rather engage in alternate mechanisms such as becoming less sociable when infected (Bos *et al.*, 2012), limiting their use of nest space (Quevillon *et al.*, 2015; Stroeymeyt *et al.*, 2018), interacting primarily with other foragers (Mersch *et al.*, 2013; Stroeymeyt *et al.*, 2018) or even leaving the nest when facing death (Chapuisat, 2010; Heinze & Walter, 2010). Nest workers are, however, less expendable. If pathogens reach nest workers, it is not surprising that they swiftly act to remove pathogens in order to protect the colonies young work force, the brood and queen. If an infection does reach as far as the brood, ants have been shown to remove (Ugelvig *et al.*, 2010) and destructively disinfect any infected brood (Pull *et al.*, 2018).

Our model simulations were built using data collected from our previous work on experimentally established colonies, which are more representative of incipient or young colonies. However, most ant colonies grow to sizes substantially larger than 100 individuals. Thus, whilst these patterns may hold for small and incipient colonies they may not hold for larger colonies and therefore we should express caution when generalising these findings. Moreover, our study did not consider the potential effects of spatial use (Stroeymeyt *et al.*, 2014; Quevillon *et al.*, 2015) or chemical control (Fernández-Marín *et al.*, 2006; Poulsen *et al.*, 2006) and may not accurately represent all colony-level effects. *Anoplolepis custodiens* is expected to rely on both these mechanisms, with our previous work showing that foragers do not utilise the same space as brood and queens (Chapter 4). Further, there is some support for

chemical control as a mechanism of conidia control in two of the assessed species, as we have identified potential group-level disinfectant behaviours in *C. fulvopilosus* and *A. custodiens*. Finally, for ease of model implementation our study used proportional grooming effects based on our previously collected work; however, we expect that this may not accurately represent the rates of grooming, which may occur in a time or dose dependent manner. Consequently, future work should assess grooming efficacy over a shorter time span in order to improve our understanding of conidia removal rates as a function of time and conidia load. Further, we contend that more work should be conducted on assessing secondary transfer rates (but see Konrad *et al.*, 2012) to determine whether they are uniform throughout colonies or whether they alter in a dose dependent manner. Additionally, future modelling studies may do well to utilise individual based methods to accurately represent factors such as chemical control, spatial use and activity levels.

Through the implementation of matrix projection models, we showed that species differ in their efficiency in managing exposure to entomopathogenic fungi. Yet, all species under standard conditions were able to manage their conidia exposure within the window of poor conidia adherence prior to conidia germination. In light of grooming behaviour, self-grooming was the primary mechanism for controlling conidia in two of the species *C. fulvopilosus* and *T. sericeiventre* but also enhanced the rate of conidia removal in *A. custodiens*. Allogrooming was the primary mechanism for conidia management in *A. custodiens* while providing increased rates of conidia control in *T. sericeiventre;* However, allogrooming was not effective in simulations of *C. fulvopilosus*. Finally, we showed that species took longer to manage conidia when more individuals were exposed, but if exposure was limited to foragers conidia persisted for longer than in colonies were exposure occurred throughout. Overall, our study showed that by utilising matrix projection models based on empirical data, we could facilitate the assessment of experimentally difficult questions, such as excluding self-grooming from pathogen defence, to better understand the mechanisms of managing exposure to fungal pathogens in ants.

Chapter 6: Research contributions

DJP and CH, with aid from TCW, conceived and designed the mathematical model. CH and DJP implemented the model in the Mathematica framework. DJP modified, processed and analysed all model runs and subsequent analysis. DJP wrote the paper with input from TCW and CH.

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Chapter 7: General Discussion:

Social insects and ants in particular are extremely successful the world over (Wilson, 1990), reaching large colony sizes and exploiting novel niches. However, despite sociality providing numerous benefits, there are associated costs that come with individuals living in large, closely related social groups, namely an increase in disease risk (Hölldobler & Wilson, 1990). Social insects exploit behaviours at the individual level, to combat disease risks, that benefit the colony. Collectively these mechanisms have been termed social immunity (SI; Cremer *et al.*, 2007). Social immunity encompasses a range of mechanisms, with species adopting those mechanisms more suited to the control of their particular pathogens. Ants, as predominantly soil living organisms, are expected to primarily encounter fungal pathogens (Boomsma *et al.*, 2005) and consequently invest in SI mechanisms that mitigate the effects of these soil-borne fungal pathogens. Furthermore, pathogen pressure is expected to vary across ecosystems, thus an ant's ecology and life history will affect which pathogens they frequently encounter and develop defences against. We assessed a range of SI mechanisms in three species of soiling-dwelling ants, *Anoplolepis custodiens, Camponotus fulvopilosus* and *Tetramorium sericeiventre,* to determine their ability to mitigate exposure to a generalist entomopathogenic fungi. Exposure to these fungi is characterised by a period of opportunity during which ants can engage in social immunity behaviours, before fungal conidia can germinate, this period ranges from 0-48 hours (Vestergaard *et al.*, 1999). We found that all three species were able to mitigate exposure to the fungi, which was accomplished using different strategies in each species.

Of the three species, A. *custodiens* adopted the widest range of social immunity mechanisms, which facilitated the efficient mitigation of pathogen exposure in simulations (Chapter 6) and in experimental trials (Chapter 2). *Anoplolepis custodiens* appear to rely primarily on group level mechanisms for the control of pathogens and are characterised by extremely high interaction rates. These interaction rates are plastic, reacting to group size by increasing as group size increases (Chapter 2) and to pathogen exposure by upregulating the frequency of allogrooming and downregulating trophallaxis (Chapter 3) when in contact with a pathogen. They appear to rely on allogrooming as their primary mechanism for conidia control (Chapter 6). Altered allogrooming in response to pathogen exposure is common in assessments of social immunity, with ants removing pathogens before infections can develop (Hughes *et al.*, 2002; Walker & Hughes, 2009; Bos *et al.*, 2012; Okuno *et al.*, 2012). *Anoplolepis custodiens* did not only rely on grooming to mitigate pathogen exposure but showed prophylactic spatial use

patterning or organisational immunity (Naug & Smith, 2007; Stroeymeyt *et al.*, 2014) to limit pathogen exposure and further, when exposed to a pathogen, altered these spatial patterns to limit pathogen spread (Chapter 4). However, *A. custodiens* showed relatively low levels of self-grooming, even though they appear to benefit from it they don't seem to rely solely on it (Chapter 6). We contend that *A. custodiens* rather than rely solely on self-grooming, has instead come to rely on alternate mechanisms to control pathogens. These may include behavioural modifications such as, effective use of nest space, high interaction rates and potential chemical control of pathogens. We surmise that their wide range of pathogen control mechanisms allow them to manage a range of pathogens and this may play a role in their ecological dominance in their native range (Addison & Samways, 2000, 2006; Keiser *et al.*, 2015). In addition, their extensive polydomous nesting behaviour (personal observations), necessitates reliance on group level behaviours, such as high levels of allogrooming to maintain colony odour through the transfer of hydrocarbons (Soroker *et al.*, 1994, 1995; Sturgis & Gordon, 2012) and subsequently this behaviour has been co-opted to mitigate pathogen risks.

In contrast to *A. custodiens'* reliance on group level behaviours, *C. fulvopilosus* was found to rely primarily on individual level response to pathogen exposure. They relied on effective self-grooming (Chapter 2 and 6) and, when exposed to pathogens, decreased the number of interactions in the nest (Chapter 5). They were characterised by extremely low rates of interaction (Chapter 2 and 3) and allogrooming which was ineffective in mitigating pathogen exposure (Chapter 2 and 6). This lack of reliance on allogrooming was surprising given its importance in other species for mitigating fungal exposure (Rosengaus *et al.*, 1998; Hughes *et al.*, 2002; Morelos-Juárez *et al.*, 2010; Reber *et al.*, 2011; Okuno *et al.*, 2012; Qiu *et al.*, 2014); however, other studies have found that some species rely on self-grooming in response to pathogen exposure over allogrooming (Tranter *et al.*, 2014; Bos *et al.*, 2019). Furthermore, colonies of *C. fulvopilosus* do not appear to rely on prophylactic or active partitioning of nest space (Chapter 4). We expect that *C. fulvopilosus* may rely on individual-based behaviours adopting a "see to oneself first" approach, this appears consistent with their ecology as solitary foragers from monodomous nests (personal observations). Foraging workers forage alone for extended periods of time and may rather than rely on infrequent interactions with nestmate invest in individual mechanisms for pathogen control, such as self-grooming. Although, it may be more likely that they rely on alternate mechanisms for conidia control than those assessed in this study. Given their strong and well melanised cuticle, which has been associated with stronger individual immune systems (Feldhaar & Gross, 2008; José De Souza *et al.*, 2011; but

see Sinotte *et al.*, 2018), they may rely on this potential innate immunity for pathogen mitigation. We also expect that they may utilise chemical control of pathogens, given their proclivity for spraying formic acid (Robertson & Zachariades, 1997), which has been shown to have strong antimicrobial properties (Graystock & Hughes, 2011; Tragust *et al.*, 2013; Brütsch *et al.*, 2017).

Tetramorium sericeiventre was characterised by strong individual level defences in self-grooming and did not show particularly strong reliance on group level benefits, unlike *C. fulvopilosus,* they did benefit from allogrooming. Of the three species assessed *T. sericeiventre* showed the most effective self-grooming capacity (Chapter 2), which played a strong role in managing conidia in simulations (Chapter 6). When analysing how they utilised nest space we found that they did not show any prophylactic or activated spatial use patterns (Chapter 4). We correctly predicted that *T. sericeiventre* would show the greatest individual level resistance to fungal pathogens, given their preference to nest in moist soils (personal observations), where fungi are expected to proliferate. Our hypothesis stems from our pilot trials where *T. sericeiventre* ants maintained in isolation had the lowest mortality rates after pathogen exposure, which is a clear indication of their strong self-grooming capacity and was independent of any group level responses. Colonies of *T sericeiventre* represent the smallest colony sizes of the species assessed and appear to be monodomous (personal observations) as such they may have less pressure to rely on allogrooming, alternatively they may also rely on a naturally strong innate immunity resulting from melanisation (Wilson *et al.*, 2001). *Tetamorium sericeiventre* ants have an extremely broad geographic distribution across most of sub-Saharan Africa and are characterised as an "opportunistic" functional type as per Hoffmann and Andersen (2003), and coexist with the invasive Argentine ant (Luruli, 2007). Invasive species are expected to have low pathogen loads as a result of enemy release; yet, the diseases they do harbour and transfer to novel habitats may not affect their host, but could prove harmful to native species (Cremer, 2019). A possible reason for the survival of *T. sericeiventre* in the face of invasions by Argentine ants may be a result of their potentially high innate immunity and high level of self-grooming. However, this is purely speculative, and more research would need to be undertaken to confirm *T. sericeiventre* innate immunity and to determine whether the Argentine ant has introduced any novel pathogens.

Each of the three assessed species was able to mitigate exposure to a generalist fungal pathogen; but, they each utilised a different combination of SI mechanisms. This reflects the importance of assessing pathogen control via a range of methods. By assessing a range of mechanisms previously explored in other studies, we were able to explore variation in mechanisms for pathogen control. We assessed grooming (Reber *et al.*, 2011; Okuno *et al.*, 2012), spatial use patterns (Baracchi & Cini, 2014; Quevillon *et al.*, 2015) and network dynamics (Mersch *et al.*, 2013; Stroeymeyt *et al.*, 2018) using group level observations, colony level recordings and mathematical models. Our work, together with a growing body of literature (Hughes *et al.*, 2002; Walker & Hughes, 2011; Tranter *et al.*, 2014, 2015; Tranter & Hughes, 2015; Bos *et al.*, 2019), shows that it is important to assess how different species manage pathogen. Particularly, as their life history and ecology play an important role in shaping these mechanisms. By combining a multispecies approach, with assessments of multiple social immune mechanisms, we were able to identify interesting differences between species and even identify potentially novel mechanisms for conidia control. Specifically, we found that species may differ in their reliance on self-grooming and allogrooming, showing the importance of studies accounting for both the effect of self-grooming and allogrooming, as opposed to merely group level responses. Furthermore, previous work has shown that organisational immunity is prevalent in social insects (Stroeymeyt *et al.*, 2014 and references within, 2018; Quevillon *et al.*, 2015); however, we found that only one of the assessed ant species relied on organisational immunity. Finally, we identified a potentially novel mechanism of pathogen control, collective disinfection, where an individual grooming her acidopore will trigger surrounding nestmates to surround the initiator and groom their. More work is required to explore this behaviour and its potential antifungal properties, since we have observed its occurrence multiple times in two of the assessed species *A. custodiens* and *C. fulvopilosus.*

In this study all three species were able to overcome exposure to a generalist entomopathogenic fungus. This may, however, represent a mismatch in selective pressures between pathogen and host (Loreto & Hughes, 2016; Cremer *et al.*, 2018). Generalist fungi can infect a range of hosts and rather than investing in overcoming the ants' social immune system may rather focus on ensuring they infect a range of hosts. Ants frequently encounter (Keller *et al.*, 2003; Hughes *et al.*, 2004; Reber & Chapuisat, 2012) and evolve defences against these pathogens. Specialist fungi on the other hand are expected to engage in an active arms race with their host, striving to overcome or evade the social immune system of ant colonies. Studies which assessed the

prevalence of specialist fungi across a range of hosts (Evans *et al.*, 2011; Araújo *et al.*, 2018) and over time within a single host (Loreto *et al.*, 2014) suggest that many ant species are under constant exposure to a specialist fungal pathogen. Future research should assess a range of social immune mechanisms across multiple species to explore how ants respond to both generalist and specialist fungal pathogens. These studies could assess whether species engaged in active arms races with specialist fungi are able to co-opt their specialised defences to mitigate exposure to generalist pathogens. An important consideration for future research in SI is to assess the importance of contamination site, studies utilise a range of exposure methodologies which may alter the investment of hosts in different mechanisms. Our study utilised a uniform spray which provides even coverage, this may have increased the importance of self-grooming as conidia could land all over the hosts body including their legs which are easily self-groomed.

Our research was limited by a distinct lack of knowledge of the ecology of ants in Southern Africa. The manual assessment of all observations further limited how much data were able to be collected and future work should prioritise automated tracking of colonies. Furthermore, during experimentation, we did not measure and specifically control the relative humidity. A high relative humidity facilitates conidia germination and so future work should more explicitly measure this. During experimentation we did account for relative humidity by providing all experimental setups with a source of water in the form of a ball of soaked cotton wool to raise relative humidity. We expect that our interventions were sufficient as conidia germination did occur, following our video recordings of nests we found colonies that were pathogen treated did succumb to an *M. anisopliae* infection although the degree was not quantified. Furthermore, in our pilot trials 91% of mortality occurred as a result of *M, anisopliae* infection, suggesting that our experimental humidity was indeed sufficient. Although, germination was not explicitly measured in each experiment.

This study was the first on social immunity in South African ants. Future work should include more species and focus on a wider range of social immunity mechanisms. An exciting possibility is to delve further into the observed collective disinfection behaviour evident in two of our species. Future work assessing network responses via automated tracking may allow for a deeper assessment of organisational immunity across the three assessed species and any other species included in future studies. Overall, through the assessment of multiple mechanisms of social immunity across three species we showed that each species is able to manage exposure

to generalist entomopathogenic fungi, through a unique combination of social immune mechanisms.

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Supplementary materials:

Supplementary tables:

Table S 2.1: GLMM results from Local Density Experiment, assessing the effects of group size(5,10 and 25 compared to 2), sanitary behaviour (D: allogrooming donated, F:Trophallaxis, S:Selfgrooming compared to allogrooming received), treatment (E: Pathogen exposed compared to Tween treated), species (CF: *Camponotus fulvopilosus*,TS: *Tetramorium sericeiventre* compared to *Anoplolepis custodiens)*, day (Day 1 compared to Day 2) and the interaction between sanitary behaviour and group size on the frequency of sanitary interactions (counts). * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero indicate NS.

	Estimate	*Std. Error*	*z value*	*Pr(>|z|)*	
Intercept	-0.45980	0.12718	-3.615	0.000300	***
Group 5	0.43185	0.13054	3.308	0.000939	***
Group 10	0.57665	0.12759	4.519	6.20e-06	***
Group 25	0.76881	0.12358	6.221	4.93e-10	***
Behaviour D	-0.80042	0.17866	-4.480	7.46e-06	***
Behaviour F	-1.16746	0.20358	-5.735	9.77e-09	***
Behaviour S	1.51853	0.11352	13.377	< 2e-16	***
Treatment E	0.08762	0.03805	2.303	0.021299	*
Species CF	-0.24948	0.04552	-5.480	4.24e-08	***
Species TS	-0.28002	0.04704	-5.952	2.64e-09	***
Day	-0.57984	0.03959	-14.647	< 2e-16	***
Group 5: Behaviour D	-0.77788	0.26440	-2.942	0.003261	**
Group 10: Behaviour D	-0.34018	0.23672	-1.437	0.150703	
Group 25: Behaviour D	-0.94302	0.25144	-3.751	0.000176	***
Group 5: Behaviour F	0.17136	0.25639	0.668	0.503898	
Group 10: Behaviour F	0.19467	0.25053	0.777	0.437128	
Group 25: Behaviour F	0.20435	0.24236	0.843	0.399120	
Group 5: Behaviour S	-0.25688	0.14795	-1.736	0.082525	
Group 10: Behaviour S	-0.30884	0.14484	-2.132	0.032978	*
Group 25: Behaviour S	-0.56639	0.14179	-3.995	6.48e-05	***

Table S 2.2: GLMM results from Local Density Experiment, assessing the effects of group size assessing the effects of group size(5,10 and 25 compared to 2), sanitary behaviour (D: allogrooming donated, F:Trophallaxis, S:Selfgrooming compared to allogrooming received), treatment (E: Pathogen exposed compared to Tween treated), species (CF: *Camponotus fulvopilosus*,TS: *Tetramorium sericeiventre* compared to *Anoplolepis custodiens)*, day (Day 1 compared to Day 2) and the interaction between sanitary behaviour and group size on the duration of sanitary behaviours (in seconds). * represent significance at $p < 0.05$, ** represent significance at $p < 0.01$, *** represent significance at $p < 0.001$ whilst lines crossing zero indicate NS.

	Estimate	*Std. Error*	*z value*	*Pr(>|z|)*	
Intercept	3.51815	0.13645	25.783	< 2e-16	***
Group 5	0.50564	0.13195	3.832	0.000127	***
Group 10	0.69730	0.12906	5.403	6.56e-08	***
Group 25	0.90123	0.12559	7.176	7.18e-13	***
Behaviour D	-0.81305	0.17925	-4.536	5.74e-06	***
Behaviour F	-1.19820	0.20408	-5.871	4.33e-09	***
Behaviour S	1.52518	0.11681	13.057	< 2e-16	***
Treatment E	0.10357	0.04132	2.506	0.012198	*
Species CF	-0.04900	0.04901	-1.000	0.317407	
Species TS	-0.33027	0.05212	-6.337	2.34e-10	***
Day	-0.68091	0.04266	-15.963	< 2e-16	***
Group 5: Behaviour D	-0.87222	0.26530	-3.288	0.001010	**
Group 10: Behaviour D	-0.45435	0.23781	-1.911	0.056058	
Group 25: Behaviour D	-1.08387	0.25261	-4.291	1.78e-05	***
Group 5: Behaviour F	0.12219	0.25727	0.475	0.634831	
Group 10: Behaviour F	0.11171	0.25154	0.444	0.656961	
Group 25: Behaviour F	0.12866	0.24359	0.528	0.597368	
Group 5: Behaviour S	-0.31914	0.15342	-2.080	0.037512	*
Group 10: Behaviour S	-0.41408	0.15038	-2.754	0.005896	**
Group 25: Behaviour S	-0.76027	0.14817	-5.131	2.88e-07	***

Table S 3.1: GLMM results for model assessing the effects of Species (CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre* compared to *Anoplolepis custodiens*) and treatment (C: treatment with Tween20 or E: fungal challenge with *Metarhizium anisopliae* compared to no treatment) as well as the interaction between them on the frequency of allogrooming.

Frequency Allogrooming	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.8747	0.1179	32.87	≤ 0.0001 ***
TreatmentC	0.2619	0.04472	5.86	≤ 0.0001 ***
TreatmentE	0.4471	0.04304	10.39	≤ 0.0001 ***
SpeciesCF	-2.2846	0.1947	-11.73	≤ 0.0001 ***
SpeciesTS	-0.4246	0.1685	-2.52	0.01175 *
TreatmentC:SpeciesCF	0.3936	0.1394	2.82	0.00474 **
TreatmentI:SpeciesCF	-0.001575	0.1478	-0.01	0.9915
TreatmentC:SpeciesTS	-0.09633	0.07189	-1.34	0.1803
TreatmentI:SpeciesTS	-0.4251	0.07243	-5.87	≤ 0.0001 ***

Table S 3.2: GLMM results for model assessing the effects of Species (CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre* compared to *Anoplolepis custodiens*) and treatment (C: treatment with Tween20 or E: fungal challenge with *Metarhizium anisopliae* compared to no treatment) as well as the interaction between them on the duration of allogrooming.

Duration Allogrooming	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.9620	0.08033	49.32	≤ 0.0001 ***
TreatmentC	0.0009784	0.006206	0.16	0.8747
TreatmentE	-0.01831	0.006005	-3.05	0.002293 **
SpeciesCF	-0.003284	0.1144	-0.03	0.9771
SpeciesTS	0.5400	0.1136	4.75	≤ 0.0001 ***
TreatmentC:SpeciesCF	0.4972	0.01794	27.71	≤ 0.0001 ***
TreatmentI:SpeciesCF	0.4815	0.01884	25.56	≤ 0.0001 ***
TreatmentC:SpeciesTS	-0.03091	0.008631	-3.58	≤ 0.0001 ***
TreatmentI:SpeciesTS	-0.1723	0.008861	-19.44	≤ 0.0001 ***

Table S 3.3: GLMM results for model assessing the effects of Species (CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre* compared to *Anoplolepis custodiens*) and treatment (C: treatment with Tween20 or E: fungal challenge with *Metarhizium anisopliae* compared to no treatment) as well as the interaction between them on the frequency of trophallaxis.

Frequency Trophallaxis	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.2562	0.1002	32.50	≤ 0.0001 ***
TreatmentC	-0.09087	0.06662	-1.36	0.1726
TreatmentE	-0.2626	0.06981	-3.76	≤ 0.0001 ***
SpeciesCF	-0.6936	0.1490	-4.65	≤ 0.0001 ***
SpeciesTS	-1.2213	0.1587	-7.70	≤ 0.0001 ***
TreatmentC:SpeciesCF	0.1796	0.1119	1.61	0.1084
TreatmentE:SpeciesCF	0.08782	0.1188	0.74	0.4598
TreatmentC:SpeciesTS	0.3155	0.1318	2.39	0.01672 *
TreatmentE:SpeciesTS	0.2554	0.1390	1.84	0.06615.

Table S 3.4: GLMM results for model assessing the effects of Species (CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre* compared to *Anoplolepis custodiens*) and treatment (C: treatment with Tween20 or E: fungal challenge with *Metarhizium anisopliae* compared to no treatment) as well as the interaction between them on the duration of trophallaxis.

Duration Trophalaxis	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	3.6890	0.08672	42.54	≤ 0.0001 ***
TreatmentC	-0.1102	0.01087	-10.13	≤ 0.0001 ***
TreatmentE	-0.1598	0.01174	-13.62	≤ 0.0001 ***
SpeciesCF	-0.02310	0.1229	-0.19	0.8509
SpeciesTS	-0.2642	0.1234	-2.14	0.0323 *
TreatmentC:SpeciesCF	0.6199	0.01681	36.87	≤ 0.0001 ***
TreatmentE:SpeciesCF	0.7069	0.01791	39.46	≤ 0.0001 ***
TreatmentC:SpeciesTS	0.5422	0.02203	24.61	≤ 0.0001 ***
TreatmentE:SpeciesTS	0.6380	0.02299	27.75	≤ 0.0001 ***

Table S 3.5: Mean and standard deviation (StD) for the frequency and duration of interactions in of three species of ants (AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* or TS: *Tetramorium sericeiventre*) across treatment (Baseline: no treatment, control: treatment with Tween20 or Exposure: fungal challenge with *Metarhizium anisopliae*).

Species	Treatment	Mean allogrooming duration (s)	StD allogrooming duration (s)	Mean trophallaxis duration (s)	StD Trophallaxis duration (s)	Mean allogrooming frequency	StD allogrooming frequency	Mean trophallaxis frequency	StD trophallaxis frequency
AC	B	51.50	65.06	39.69	74.57	49.17	25.79	26.22	11.93
	C	51.90	60.02	35.74	57.90	63.89	21.93	23.94	8.73
	E	49.54	62.45	31.54	51.54	76.89	28.51	20.17	8.84
CF	B	54.07	71.00	39.32	37.26	4.94	4.61	13.17	5.86
	C	85.16	78.61	64.99	66.56	9.52	6.98	14.39	5.81
	E	83.37	69.48	66.20	82.75	7.67	8.61	11.06	5.03
TS	B	90.14	100.73	30.55	36.20	32.39	20.87	7.72	5.02
	C	90.36	124.08	49.61	67.98	38.22	13.49	9.67	6.90
	E	76.08	112.22	51.20	83.78	33.11	18.99	7.67	5.55

Supplementary figures:

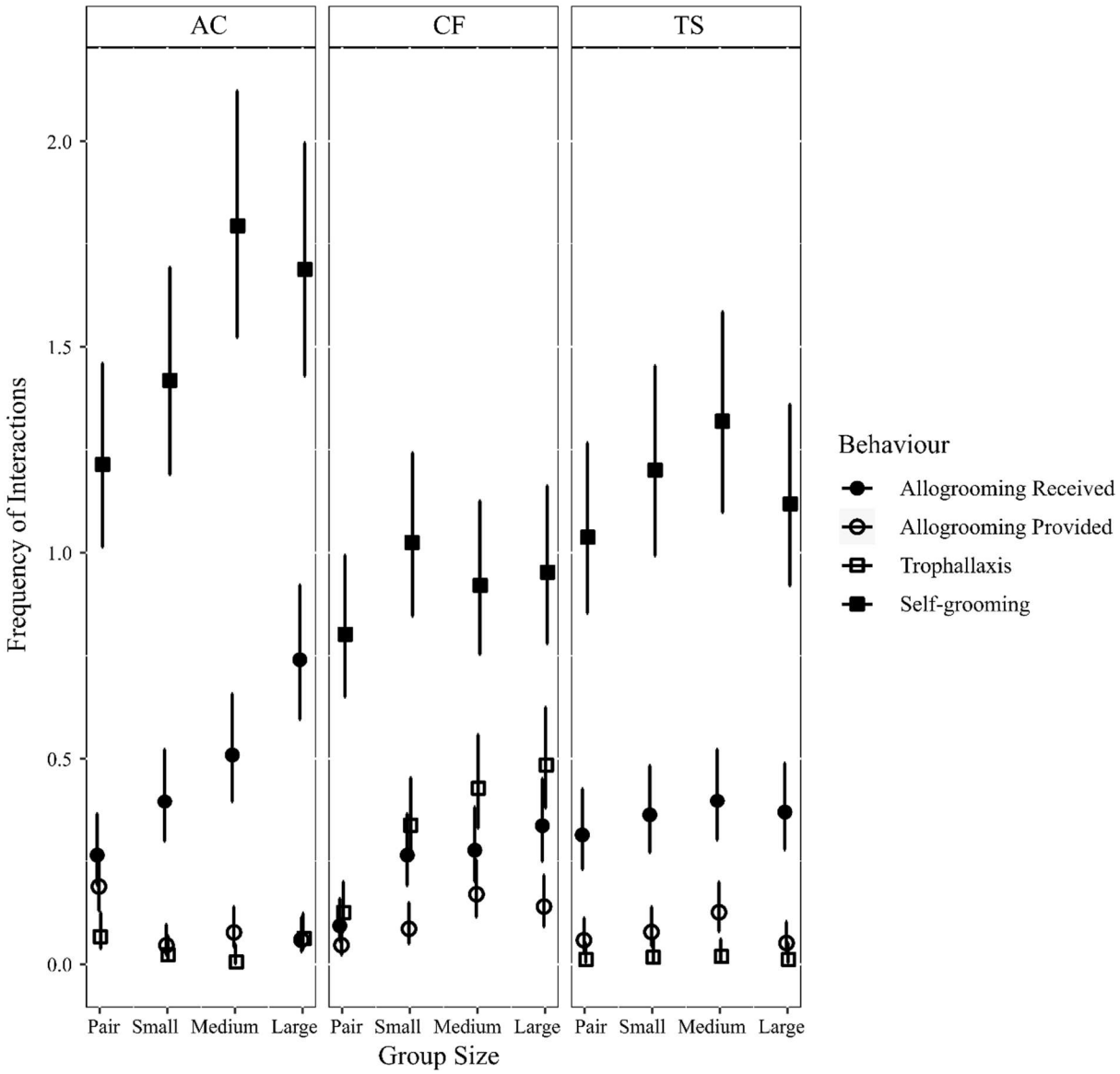

Figure S 2.1: Plot of Marginal effects and 95% CI of the interaction between local density and Sanitary behaviour on the frequency of interactions in AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* and TS: *Tetramorium sericeiventre* generated based on GLMM predictions.

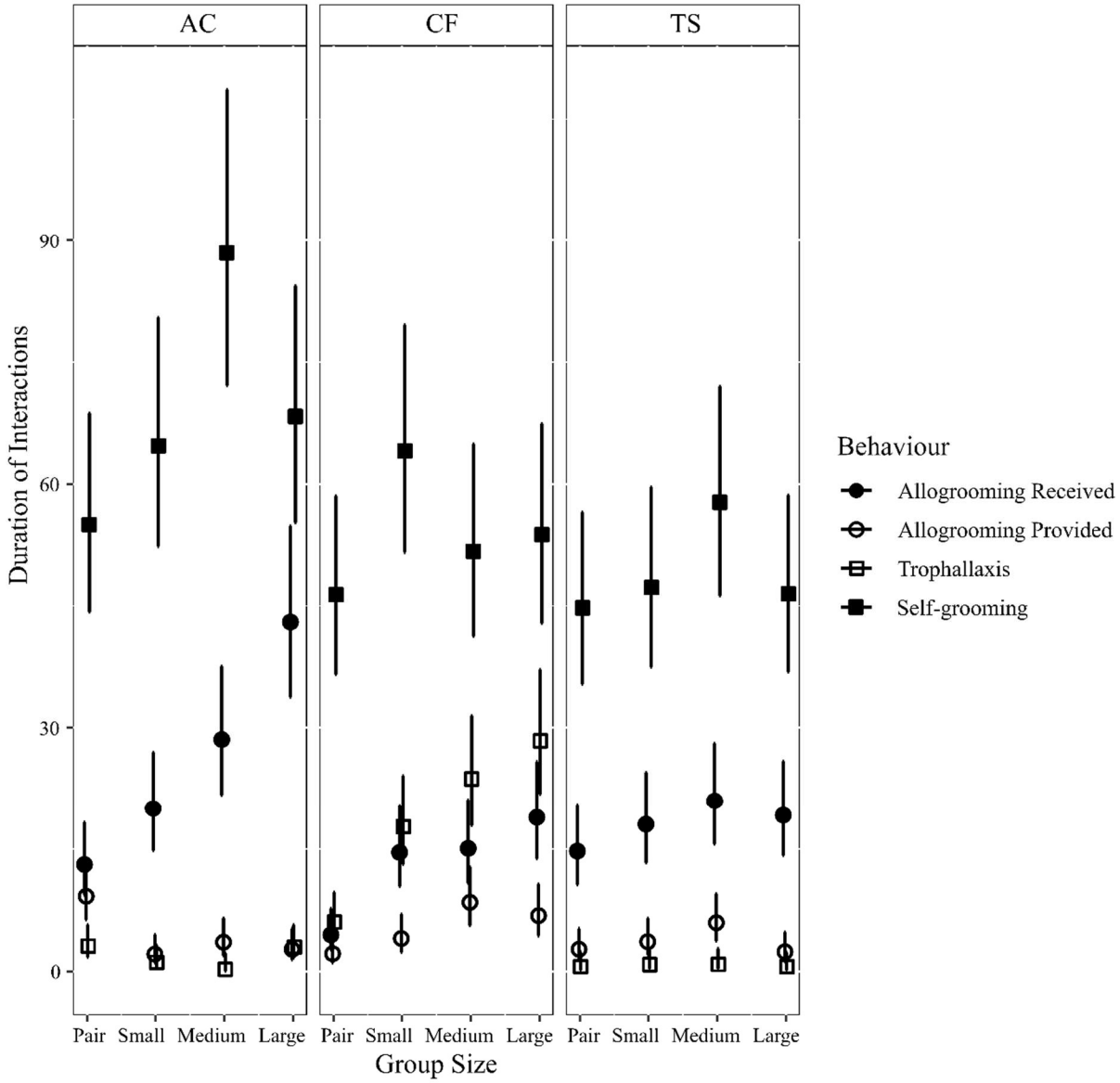

Figure S 2.2: Plot of Marginal effects and 95% CI of the interaction between local density and Sanitary behaviour on the Duration of interactions in AC: *Anoplolepis custodiens*, CF: *Camponotus fulvopilosus* and TS: *Tetramorium sericeiventre* generated based on GLMM predictions.

Figure S 3.1: Photos depicting potential group-level disinfection in *Camponotus fulvopilosus*. A: Pre-group level behaviour. B: Group level behaviour. The circled individual in frame A initiates acidopore grooming, prompting most nearby individuals to also engage in acidopore grooming as seen in frame B. This represents a potential first description of group level disinfection.

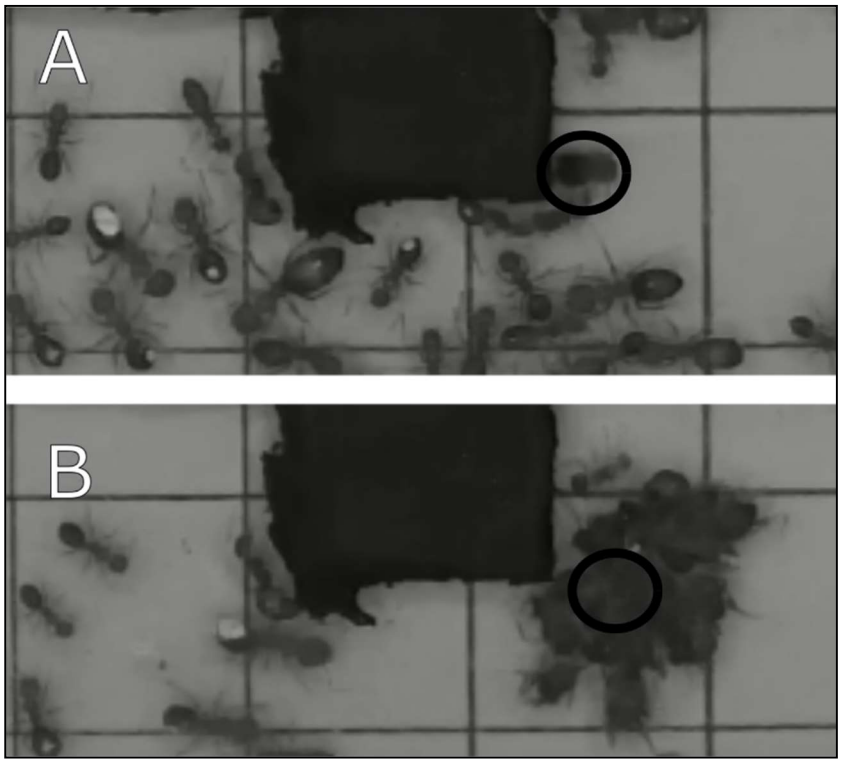

Figure S 3.2: Photos depicting potential group-level disinfection in *Anoplolepis custodiens*. A: Pre-group level behaviour. B: Group level behaviour. The circled individual in frame A initiates acidopore grooming, prompting most nearby individuals to also engage in acidopore grooming as seen in frame B. This represents a potential first description of group level disinfection.