LIVING IN AN INFECTIOUS WORLD: HOST–PATHOGEN INTERACTIONS SHAPE SOCIAL IMMUNITY IN TERMITES

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This dissertation is dedicated to my family.

To the most important people in my life:

To my parents, Fabiola Mora and Jairo Esparza, for all the love, encouragement and trust.

To my sister, Silvia Esparza Mora, for her invaluable company, love and friendship.

I love you all and could not have done this without you.

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LIST OF ABBREVIATIONS

AMP Antimicrobial peptides

PAMPs Pathogen-associated molecular patterns

PRRs Pattern recognition receptors

PGRPs Peptidoglycan recognition proteins

GNBPs Gram-negative binding proteins

PRPs Pathogen recognition proteins

Imd Immune deficiency

JAK Janus kinase

STAT Signal transducers and activators of transcription

DIF Dorsal-related immunity factor

JNK c-jun N-terminal kinase

GNBP-2 Gram-negative binding protein 2

GDL D-d-gluconolactone

CHCs Cuticular hydrocarbons

PDA Potato dextrose agar

CFU Colony-forming unit

LPS Lipopolysaccharides

PO Phenoloxidase

gfp Green fluorescent protein gene

GFS Glufosinate ammonium

CME Chlorimuron ethyl

LiAc Lithium acetate

ssDNA Single-stranded carrier DNA

PEG Polyethylene glycol

SDNG Synthetic – defined – nitrate – glucose

ALS Acetolactate synthase

CRISPR/Cas9 Clustered regularly interspaced short palindromic re-peat/associated protein system

SUMMARY

Eusocial insect colonies can function like a single organism, a trait sometimes referred to as "superorganism". Although superorganisms greatly benefit from division of labor, they may be susceptible to infectious diseases due to increased opportunities for pathogen transmission. The mechanisms behind insect societies' ability to successfully defend against infectious diseases are of significant interest in evolutionary biology. Next to their innate immune systems, eusocial insects such as termites boast sophisticated collective defences, termed social immunity, which are thought to have evolved in response to selective pressure from pathogens. Social immunity comprises a multi-layer assembly of physiological and behavioural adaptations which effectively supress the probability of exposure and transmission of infectious diseases within a colony. Although a significant body of research has explored the broad repertoire and effectiveness of termite collective defences, the underlying mechanisms of termite social immunity remain largely unexplored. This thesis exploits termite-pathogen step-wise infection dynamics to investigate the underlying mechanisms of collective defence against different entomopathogenic agents, as well as explore the link between innate defences and collective behaviours to offset increased disease pressure. The thesis is primarily based on research conducted in the eastern subterranean termite *Reticulitermes flavipes* and the entomopathogenic fungus *Metarhizium anisopliae*; a natural host–pathogen system.

In **Chapter I**, we seek to explore the underpinnings of the "care-kill dichotomy" in termite social immunity. The care-kill switch represents a generalized social immune response found in many advanced insect societies, whereby compromised nestmates are cared for when possible but sacrificed if necessary. In the case of fungal infection, we ask whether termites can detect and respond to infected individuals when pathogens (or infectious conidia) are absent from their cuticle; the rationale being to experimentally manipulate the type of cues that are presented to interacting nestmates. In this chapter, we sought to identify potential triggers for the switch from sanitary care to elimination defence (cannibalism), which represents the so-called care-kill transition. We sought to demonstrate how *R. flavipes* termites detect and respond to internal *M. anisopliae* infection at different stages of infection progression, as well as test the effect of pathogen viability on social immunity. By injecting fungal blastospores directly into the hemocoel of individuals, we removed the pathogen as a direct cue that could be detected by responding nestmates. Injection, regardless of blastospore viability, led to slightly increased rates of grooming, but also rapid transition to cannibalism (even at early stages of an internal infection), particularly when termites became visibly moribund following injection with viable blastospores. Surprisingly, however, cannibalism was still observed when termites were injected with dead blastospores and were not terminally ill, indicating that the threshold at which elimination behaviour is triggered can be reached at a very early stage during internal *M. anisopliae* infection, before viability or even terminal disease status is known. The faster cannibalism response to viable blastospore-injected termites could be due to the active synthesis of virulence factors from the pathogen

such as destruxins, although this remains to be tested. Since termites may be expected to communicate with their nestmates via chemical compounds called cuticular hydrocarbons (CHCs), alterations in the CHCs profile associated with internal fungal pathogen presence, could represent important signals for responding nestmates. We used gas chromatography-mass spectrometry (GC–MS) in the second part of Chapter I to identify cues associated with disease status and explore potential CHC signal(s) that may be responsible for triggering elimination behaviours. We found that CHC profiles were significantly altered in individuals injected with viable but not dead blastospores, and at 15 but not at 12 hours post infection. More specifically, we detected significant increases in four exclusively methyl-branched CHCs 15 hours after injection with viable blastospores compared to control-injected individuals, which we speculate could be correlated with the advanced state of moribundity of these challenged individuals, and which may contribute as possible chemical cues triggering the high levels of observed cannibalism. Although a direct link between the identified CHC compounds and altered social immune behaviour remain to be tested, these data suggest that termites could employ chemical signals provoked by early internal immune activation to trigger cannibalism.

In **Chapter II** we expand on the role of social immunity against potentially novel infection threats, as well as further explore the conditions that trigger specific behavioural defences. To understand the interaction between *R. flavipes* and the non-native entomopathogenic bacterium *Pseudomonas entomophila* and compare termites' collective defences between a native fungal entomopathogen and a non-native bacterium as well as characterize associated cuticular hydrocarbon (CHC) changes. We injected termites with different doses of either viable / dead *P. entomophila* or viable / dead *M. anisopliae* blastospores. As expected, injection regardless of severity and pathogen type led to slightly increased rates of grooming but rapid transition to cannibalism. Cannibalism was particularly evident when infected individuals were injected with high doses of viable *P. entomophila* and *M. anisopliae* blastospores (causing 100% mortality). Such individuals showed external signs of disease and were close to death, but as described in Chapter I, cannibalism was still exhibited following injection with dead blastospores, which elicited only 40% mortality. Interestingly, injection with an equivalent dose of viable *P. entomophila* (causing 40% mortality) did not elicit similarly elevated amounts of cannibalism, suggesting that triggers stimulating elimination behaviours may be pathogen-specific in this termite. We hypothesize that termites may have evolved greater sensitivity to fungal versus non-native bacterial infection, due to the likely long evolutionary association with the former. These results nevertheless show that collective defences of *R. flavipes* are effective against both fungal and bacterial challenges, with elimination behaviours being transferable to diverse and potentially novel infection threats (like *P. entomophila*). Analysis of CHC profiles from termites injected with different bacterial and fungal doses revealed unique patterns of CHCs, with a discriminant analysis showing a particularly distinct profile in termites injected with viable blastospores − treatment associated with the strongest cannibalism response.

In **Chapter III**, we examined the inhibition effect of the external antifungal activity of the Gram-negative binding protein 2 (GNBP-2) and collective behaviours after exposure of single individuals to *M. anisopliae*. Termites may depend on the innate immune system for defence against pathogens. Therefore, immune effectors have been co-opted from an internal role to an external role to prevent infection from entomopathogenic fungi that can evade innate immune defences after penetration of the cuticle. Termicins and GNBP-2 associated β-1,3-glucanase activity are involved in external defence against *M. anisopliae*. The effectiveness of this external defence strategy likely depends on or is bolstered by collective behaviours. Through the suppression by an inhibitor (D-d-gluconolactone (GDL)) of the termite GNBP-2 β-1,3-glucanase activity that is capable of degrading entomopathogenic fungi, we found that collective defences such as grooming are not triggered, but instead cannibalistic behaviours are reduced. This suggests that the internal immune system or the use of antimicrobial secretions may be linked to certain collective immune behaviours in termites. Understanding the molecular basis of termite defence mechanisms may also be relevant for the development of sustainable control strategies against pest termite species.

Finally, on the side of the pathogen, we require efficient transformation methods for entomopathogenic fungi to develop essential molecular tools for elucidating the function of genes involved in fungus-insect interactions. For example, by inserting or deleting genetic elements in the genome of the strain of interest, it is possible to modify the expression of targeted endogenous genes, and thereby experimentally test their role in the pathogen's infection strategy, and how they may influence host fitness, immunity, and ultimately, social immune traits. However, developing methods to deliver foreign nucleic acid into fungal cells represents a major stumbling block in fungal genetics. In **Chapter IV** we address this issue by testing suitable selection markers: glufosinate ammonium (GFS) and chlorimuron ethyl (CME) for transformation of *M. anisopliae* with green fluorescence protein gene (*gfp*). Since *M. anisopliae* can produce blastospores through yeast-like budding in liquid culture, as well as being thin-walled and unicellular, an efficient blastospore-based transformation system was developed for the introduction of "*bar-gfp*" and "*sur-gfp*" constructs using the LiAc/ssDNA/PEG method. These genetic constructs conferred resistance to GFS and CME, respectively. We efficiently achieved integration of these genes into the fungal genome, resulting in resistant transformants, MA-0001 (*baR-gfp*) and MA-0002 (*suR-gf*p), which expressed high levels of green fluorescence in conidia, hyphae, blastospores, as well as on termite cadavers after injection of blastospores within the host hemolymph. The generation of these stable MA-0001 and MA-0002 strains allows us to monitor the internal infection process that begins after the fungus penetrates the cuticle and proliferates inside the termite hemolymph as hyphal bodies. This research represents a proof-of-principle that the genetic manipulation of *M. anisopliae* for studying gene function and for elucidating the relevant factors for pathogenic interactions with insects, is feasible. Targets for future work could include the generation of strains lacking virulence factors such as destruxins and examining the individual and social immune consequences of infection with genetically modified fungi.

The present work highlights the importance of the effective innate immune responses in addition to physiological and behavioural defences in the termite *R. flavipes*, and how these are mediated by precise chemical communication which also contribute to social immunity. This allows *R. flavipes* to be less susceptible to pathogen infections and facilitates its evolutionary success.

Keywords: *Reticulitermes flavipes*, *Metarhizium anisopliae*, *Pseudomonas entomophila*, host–pathogen interactions, individual immunity, social immunity, care-kill strategy.

ZUSAMMENFASSUNG

Eusoziale Insektenkolonien können wie ein einzelner Organismus funktionieren, eine Eigenschaft, die manchmal als „Superorganismus“ bezeichnet wird. Obwohl Superorganismen stark von der Arbeitsteilung profitieren, können sie aufgrund der erhöhten Möglichkeiten zur Übertragung von Krankheitserregern anfällig für Infektionskrankheiten sein. Die Mechanismen hinter der Fähigkeit von Insektengesellschaften, sich erfolgreich gegen Infektionskrankheiten zu verteidigen, sind von großem Interesse für die Evolutionsbiologie. Neben ihrem angeborenen Immunsystem verfügen eusoziale Insekten wie Termiten über ausgeklügelte kollektive Abwehrkräfte, die als soziale Immunität bezeichnet werden und sich vermutlich als Reaktion auf den selektiven Druck von Krankheitserregern entwickelt haben. Die soziale Immunität umfasst eine vielschichtige Ansammlung von physiologischen und Verhaltensanpassungen, die die Wahrscheinlichkeit einer Exposition und Übertragung von Infektionskrankheiten innerhalb einer Kolonie wirksam unterdrücken. Obwohl eine beträchtliche Anzahl von Forschungsarbeiten das breite Repertoire und die Wirksamkeit der kollektiven Verteidigung von Termiten untersucht hat, bleiben die zugrunde liegenden Mechanismen der sozialen Immunität von Termiten weitgehend unerforscht. Diese Dissertation nutzt die schrittweise Infektionsdynamik von Termiten-Pathogenen, um die zugrunde liegenden Mechanismen der kollektiven Abwehr gegen verschiedene entomopathogene Erreger zu untersuchen, sowie die Verbindung zwischen angeborener Abwehr und kollektivem Verhalten zu untersuchen, um einen erhöhten Krankheitsdruck auszugleichen. Die Dissertation basiert hauptsächlich auf Forschungen, die in der östlichen unterirdischen Termite *Reticulitermes flavipers* und der entomopathogene Pilz *Metarhizium anisopliae* durchgeführt wurden; ein natürliches Wirt–Pathogen-System.

In **Kapitel I** versuchen wir, die Grundlagen der „Care–Kill-Dichotomie“ in Bezug auf die soziale Immunität von Termiten zu untersuchen. Der Care–Kill-Schalter stellt eine allgemeine soziale Immunantwort dar, die in vielen fortgeschrittenen Insektengesellschaften zu finden ist, wobei kompromittierte Nestgenossen nach Möglichkeit gepflegt, aber wenn nötig geopfert werden. Im Falle einer Pilzinfektion fragen wir, ob Termiten infizierte Individuen erkennen und darauf reagieren können, wenn Krankheitserreger (oder infektiöse Konidien) in ihrer Kutikula fehlen. Das Grundprinzip besteht darin, die Art von Hinweisen, die interagierenden sozialen Nestgenossen präsentiert werden, experimentell zu manipulieren. In diesem Kapitel haben wir versucht, mögliche Auslöser für den Wechsel von der sanitären Versorgung zur Eliminationsabwehr (Kannibalismus) zu identifizieren, der den sogenannten Care–Kill-Übergang darstellt. Wir wollten zeigen, wie *R. flavipes*-Termiten eine interne *M. anisopliae*-Infektion in verschiedenen Stadien des Infektionsverlaufs erkennen und darauf reagieren, sowie die Auswirkung der Lebensfähigkeit des Krankheitserregers auf die soziale Immunität testen. Indem wir Pilz-Blastosporen direkt in das Hämocoel von Individuen injizierten, entfernten wir den Erreger als direkten Hinweis, der von reagierenden Nestgenossen erkannt werden konnte. Die Injektion führte unabhängig von der Lebensfähigkeit der Blastosporen zu leicht erhöhten Lausen (Allogrooming), aber auch zu einem schnellen Übergang zum

Kannibalismus (selbst in frühen Stadien einer inneren Infektion), insbesondere wenn Termiten nach der Injektion lebensfähiger Blastosporen sichtbar sterbend wurden. Überraschenderweise wurde Kannibalismus jedoch immer noch beobachtet, wenn Termiten tote Blastosporen injiziert wurden und nicht todkrank waren, was darauf hindeutet, dass die Schwelle, bei der das Eliminationsverhalten ausgelöst wird, während einer inneren *M. anisopliae*-Infektion sehr früh erreicht werden kann, noch bevor die Lebensfähigkeit oder der Krankheitsstatus bekannt ist. Die schnellere Kannibalismusreaktion auf lebensfähige Termiten, denen Blastosporen injiziert wurden, könnte auf die aktive Synthese von Virulenzfaktoren des Pathogens wie Destruxine zurückzuführen sein, obwohl dies noch getestet werden muss. Da erwartet werden kann, dass Termiten mit ihren Nestgenossen über chemische Verbindungen kommunizieren, die als kutikuläre Kohlenwasserstoffe (CHCs) bezeichnet werden, könnten Änderungen im CHC-Profil, die mit dem Vorhandensein interner Pilzpathogene verbunden sind, wichtige Signale für reagierende Nestgenossen darstellen. Wir verwendeten Gaschromatographie-Massenspektrometrie (GC–MS) im zweiten Teil von Kapitel I, um Hinweise im Zusammenhang mit dem Krankheitsstatus zu identifizieren und potenzielle CHC-Signale zu untersuchen, die möglicherweise für das Auslösen von Eliminationsverhalten verantwortlich sind. Wir fanden heraus, dass die CHC-Profile bei Individuen, denen lebensfähige, aber nicht tote Blastosporen injiziert wurden, signifikant verändert waren, und zwar 15, aber nicht 12 Stunden nach der Infektion. Genauer gesagt haben wir 15 Stunden nach der Injektion mit lebensfähigen Blastosporen signifikante Anstiege bei vier ausschließlich methylverzweigten CHCs im Vergleich zu Kontrollinjektionen festgestellt, von denen wir spekulieren, dass sie mit dem fortgeschrittenen Sterbezustand dieser herausgeforderten Individuen korrelieren könnten und die als mögliche chemische Hinweise dienen könnten, die das hohe Maß an beobachtetem Kannibalismus auslösen. Obwohl eine direkte Verbindung zwischen den identifizierten CHC-Verbindungen und einem veränderten sozialen Immunverhalten noch getestet werden muss, deuten diese Daten darauf hin, dass Termiten chemische Signale verwenden könnten, die durch eine frühe interne Immunaktivierung hervorgerufen werden, um Kannibalismus auszulösen.

In **Kapitel II** gehen wir auf die Rolle der sozialen Immunität gegen potenziell neuartige Infektionsbedrohungen ein und untersuchen weiter die Bedingungen, die spezifische Verhaltensabwehr auslösen. Um zu verstehen wie das nicht einheimische, entomopathogene Bakterium *Pseudomonas entomophila* und *R. flavipes* sich gegenseitig beeinflussen und Vergleich der kollektiven Abwehrkräfte von Termiten zwischen einem einheimischen Pilz-Entomopathogen und einem nicht einheimischen Bakterium sowie Charakterisierung der damit verbundenen Veränderungen der kutikulären Kohlenwasserstoffe (CHC). Wir injizierten Termiten verschiedene Dosen von entweder lebensfähigen/toten *P. entomophila* oder lebensfähigen/toten *M. anisopliae* Blastosporen. Wie erwartet führte die Injektion unabhängig vom Schweregrad und Erregertyp zu leicht erhöhtem Lausen, jedoch zu einem schnellen Übergang zum Kannibalismus. Dieser war besonders evident, wenn infizierten Individuen hohe Dosen von lebensfähigen *P. entomophila* und *M. anisopliae* Blastosporen injiziert wurde, welche jeweils zu einer 100-prozentigen Sterblichkeitsrate führte. Solche

Individuen zeigten äußere Anzeichen einer Krankheit und waren dem Tode nahe, aber wie in Kapitel I beschrieben, zeigte sich nach der Injektion toter Blastosporen immer noch Kannibalismus, was nur eine Sterblichkeit von 40% hervorrief. Interessanterweise löste die Injektion mit einer äquivalenten Dosis von lebensfähigen *P. entomophila* keine ähnlich erhöhten Mengen an Kannibalismus aus. Dies deutet darauf hin das Auslöser, die das Eliminationsverhalten stimulieren bei dieser Termite erregerspezifisch sein könnten. Wir vermuten, dass Termiten aufgrund der wahrscheinlich langen evolutionären Assoziation mit Pilzen eine größere Empfindlichkeit gegenüber solchen infektionen als gegenüber nicht-nativen bakteriellen Infektionen entwickelt haben. Diese Ergebnisse zeigen dennoch, dass die kollektive Abwehr von *R. flavipes* sowohl gegen pilzliche als auch bakterielle Herausforderungen wirksam ist, wobei das Eliminationsverhalten auf verschiedene und potenziell neuartige Infektionsbedrohungen (wie *P. entomophila*) übertragbar ist. Die Analyse der CHC-Profile der Termiten, denen unterschiedliche Bakterien- und Pilzdosen injiziert wurden, ergab einzigartige Muster, wobei eine Diskriminanzanalyse ein besonders ausgeprägtes Profil bei Termiten zeigte, denen lebensfähige Blastosporen injiziert wurden - eine Behandlung, die mit der stärksten Kannibalismus-Reaktion verbunden war.

In **Kapitel III** haben wir die Hemmwirkung der externen antimykotischen Aktivität des Gram-negativen Bindungsproteins 2 (GNBP-2) und kollektive Verhaltensweisen nach Exposition einzelner Individuen gegenüber *M. anisopliae* untersucht. Termiten können zur Abwehr von Krankheitserregern auf das angeborene Immunsystem angewiesen sein. Daher wurden Immuneffektoren von einer internen Rolle zu einer externen Rolle kooptiert, um eine Infektion durch entomopathogene Pilze zu verhindern, die der angeborenen Immunabwehr nach dem Eindringen in die Kutikula entgehen können. Termicine und GNBP-2-assoziierte β-1,3-Glucanase-Aktivität sind an der externen Abwehr gegen *M. anisopliae* beteiligt. Die Wirksamkeit dieser externen Verteidigungsstrategie hängt wahrscheinlich von kollektiven Verhaltensweisen ab oder wird durch diese gestärkt. Durch die Unterdrückung der GNBP-2 β-1,3-Glucanase-Aktivität der Termiten durch einen Inhibitor (D-d-Gluconolacton (GDL)), der entomopathogene Pilze abbauen kann, fanden wir heraus, dass kollektive Abwehrmechanismen wie Lausen nicht ausgelöst werden, sondern stattdessen kannibalistische Verhaltensweisen werden reduziert. Dies deutet darauf hin, dass das interne Immunsystem oder die Verwendung antimikrobieller Sekrete mit bestimmten kollektiven Immunverhaltensweisen bei Termiten in Verbindung gebracht werden können. Das Verständnis der molekularen Grundlagen von Termitenabwehrmechanismen kann auch für die Entwicklung nachhaltiger Bekämpfungsstrategien gegen Schädlingstermitenarten relevant sein.

Schließlich benötigen wir auf der Seite des Pathogens effiziente Transformationsmethoden für entomopathogene Pilze, um wesentliche molekulare Werkzeuge zur Aufklärung der Funktion von Genen zu entwickeln, die an Pilz–Insekten-Interaktionen beteiligt sind. Durch das Einfügen oder Löschen genetischer Elemente in das Genom des jeweiligen Stamms ist es beispielsweise möglich, die Expression von gezielten endogenen Genen zu modifizieren und dadurch experimentell ihre Rolle in der Infektionsstrategie des Pathogens zu testen und wie sie

Wirtsfitness, Immunität und letztlich soziale Immuneigenschaften beeinflussen können. Allerdings stellt die Entwicklung von Methoden zur Einbringung fremder Nukleinsäuren in Pilzzellen einen großen Stolperstein in der Pilzgenetik dar. In **Kapitel IV** widmen wir uns diesem Problem, indem wir geeignete Selektionsmarker testen: Glufosinat-Ammonium (GFS) und Chlorimuron-Ethyl (CME) zur Transformation von *M. anisopliae* mit dem Grün-Fluoreszenz-Protein-Gen (*gfp*). Da *M. anisopliae* Blastosporen durch hefeähnliches Sprossen in Flüssigkultur produzieren kann, sowie dünnwandig und einzellig ist, wurde ein effizientes Blastosporen basiertes Transformationssystem zur Einführung von „*bar-gfp*“ und „*sur-gfp*“ Konstrukten unter Verwendung der LiAc/ssDNA/PEG-Methode entwickelt. Diese genetischen Konstrukte verliehen Resistenz gegen GFS bzw. CME. Wir erreichten effizient die Integration dieser Gene in das Pilzgenom, was zu resistenten Transformanten MA-0001 (*baR-gfp*) und MA-0002 (*suR-gfp*) führte, die hohe Konzentrationen an grün fluoreszierendem Protein in Konidien, Hyphen und Blastosporen exprimierten sowie an Termitenkadavern nach Injektion von Blastosporen in die Wirtshämolymphe. Die Erzeugung dieser stabilen MA-0001- und MA-0002-Stämme ermöglicht es uns, den internen Infektionsprozess zu überwachen, der beginnt, nachdem der Pilz die Kutikula durchdrungen hat und sich innerhalb der Termiten-Hämolymphe als Hyphenkörper vermehrt. Diese Forschung stellt einen Beweis des Prinzips dar, dass die genetische Manipulation von *M. anisopliae* zum Studium der Genfunktion und zur Aufklärung der relevanten Faktoren für pathogene Interaktionen mit Insekten machbar ist. Ziele für zukünftige Arbeiten könnten die Generierung von Stämmen ohne Virulenzfaktoren wie Destruxine und die Untersuchung der individuellen und sozialen Immunfolgen einer Infektion mit gentechnisch veränderten Pilzen sein.

Die vorliegende Arbeit unterstreicht die Bedeutung der wirksamen angeborenen Immunreaktionen zusätzlich zu den physiologischen und verhaltensbedingten Abwehrmechanismen bei der Termite *R. flavipes* und wie diese durch präzise chemische Kommunikation vermittelt werden, die auch zur sozialen Immunität beiträgt. Dadurch ist *R. flavipes* weniger anfällig für Infektionen mit Krankheitserregern, was seinen evolutionären Erfolg begünstigt.

Schlüsselwörter: *Reticulitermes flavipes*, *Metarhizium anisopliae*, *Pseudomonas entomophila*, Wirt–Pathogen-Interaktionen, individuelle Immunität, soziale Immunität, Care-Kill Strategie.

GENERAL INTRODUCTION

1. The evolution of eusociality and social immunity

Insects show varying levels of social organization, from independent, solitary species to highly elaborate and obligatory eusocial species with large and persistent colonies, typically characterized by cooperative brood care, presence of overlapping generations in the same colony, altruism, and pronounced reproductive division of labor (López-Riquelme & Ramón, 2010; Wilson, 1971; Wilson & Hölldobler, 2005), representing the key element of eusociality (Thorne, 1997). The phenomenon of eusociality is best known and developed in insects, especially ants, termites, some bees, and wasps (Hölldobler & Wilson, 2009), all known for their extraordinary overall ecological and evolutionary success (Wilson, 1971), raising the question of how they are able to prosper.

The shift from solitary life to eusociality poses one of the major evolutionary transitions (Szathmáry & Smith, 1995). As proposed by Wheeler back in 1911, colonies of eusocial insects are often viewed as an individual, or "superorganism" (Wheeler, 1911), capable of optimal living because they act as a single self-organized, functional, and reproductive unit (Helanterä, 2016; Hölldobler & Wilson, 2009; Pull & McMahon, 2020). In other words, selection acts primarily at the colony level (Hölldobler & Wilson, 2009). The essential elements of a superorganism are not cells or tissues, but rather individuals that act collectively and closely with each other in such harmony that actual conflicts among those individual elements are largely absent or controlled (Helanterä, 2016). Thus, in a superorganism where the "colony as a whole would benefit", certain individuals, the workers, sacrifice their reproductive potential to raise the offspring to others (Nowak et al., 2010). More specifically, the non-reproducing majority is specialized to perform distinct tasks required to maximize colony efficiency and productivity (Hölldobler & Wilson, 2009; Kennedy et al., 2017).

The emergence of superorganisms has parallels to other major evolutionary transitions, such as the evolution of multicellularity and later, the metazoan bodies (Aanen, 2018; Leonhardt et al., 2016). One clear example, would be the caste system present in eusocial insects, where reproduction is limited to a minority of fertile individuals (queens and, in termites, kings), while the large majority of the population (workers and soldiers) are largely sterile (Aanen, 2018), resulting in an obligate reproductive division of labor (Boomsma, 2013). Both castes are extremely interdependent: as the workers cannot reproduce themselves, they gain indirect fitness benefits by rearing the offspring – "an example of altruism" – while the extremely reproductively adapted queen(s) require the cooperation and the assistance of workers to survive (Cremer, 2019; Cremer & Sixt, 2009; Hughes, 2012; Pull et al., 2018). From this point of view, the disposable somatic cells of the multicellular organism are akin to workers, while germline cells resemble the reproductive individuals, the queen(s) (Aanen, 2018; Cremer, 2019; Cremer & Sixt, 2009; Leonhardt et al., 2016).

In spite of the many benefits provided by marvellous feats of cooperation – effective brood care, enhanced foraging, nest building and maintenance, and improved defence – which

favoured the evolution of eusocial insects (Cremer et al., 2007; Liu et al., 2019b), eusociality also bears major costs. Living in enclosed crowded societies of genetically related individuals that interact intimately would appear to facilitate pathogen transmission, which ultimately can lead to colony collapse, making diseases a great challenge (Cremer et al., 2018; Schmid-Hempel, 1995; Schmid-Hempel, 2017). Due to such strong pathogenic pressure, eusocial insects may confront and respond to it through their own individual immune system, but in addition, they have evolved a unique colony-level immune system (Cremer, 2019; Cremer et al., 2007; Cremer & Sixt, 2009). Particularly, colony-level immune defences entail a set of physiological and behavioural adaptations, which function to prevent and mitigate infectious diseases and are commonly referred to as "social immunity" (Cremer et al., 2007; Pull & McMahon, 2020). Unlike individual immunity, where increased immune function evolves to solely benefit the single individual, social immunity evolves for the benefit of both the individual and others, enhancing the health and survival of the entire colony (Cotter & Kilner, 2010; Cremer et al., 2007; Cremer & Sixt, 2009). Thus, disease resistance influences the evolutionary success of eusocial insects.

Social immunity has been regarded to represent an analogue to the physiological immune system of multicellular organisms (another important aspect of the parallel, Figure 1) (Cremer et al., 2018; Cremer & Sixt, 2009) that evolved when the unit of selection shifted from the individual to the colony, caused by the separation of germline and soma. In superorganisms, social immunity – an evolutionary adaptation – has evolved beyond simple cooperation and is "indispensable and essential to protect the entire reproductive unit and maximize its fitness" (Pull & McMahon 2020; Cremer et al., 2018). Since disease represent a tremendous burden on survival and reproductive success (Fefferman & Traniello, 2009), both multicellular organisms and superorganisms take special care of their highly valuable cells or individuals – germline cells or queens – so they are protected against disease or are immune privileged. To protect the reproductive unit, they effectively employ social immunity to fight pathogens and interfere with each of the three steps of disease progression (Cremer & Sixt, 2009): First, once pathogens are taken up from the environment, they are prevented from entering the body or colony; second, from establishing, developing, and replicating inside them, and third, from spreading between the body's cells or healthy colony members. Therefore, host defences might arise to break or interrupt the process at each step and consequently halt the infection (Cremer, 2019).

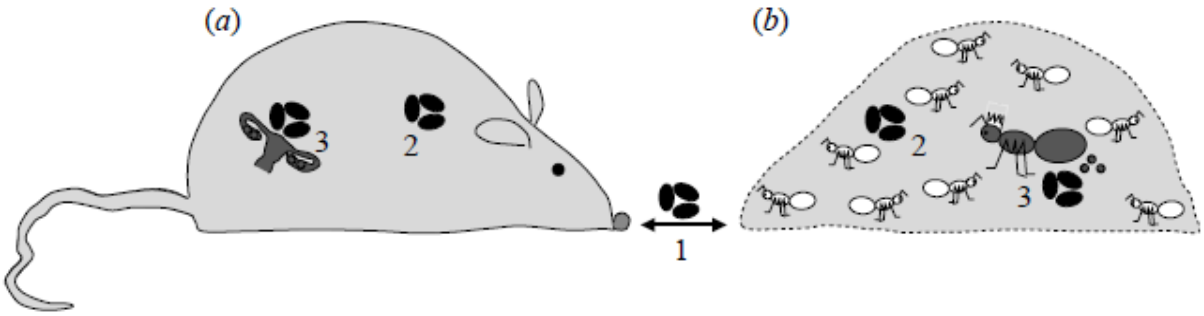

Figure 1. (a) Steps of infection and anti-pathogen defence of individual multicellular organisms and **(b)** insect colonies.

1. Border defence: pathogens (black ovals) are prevented from entering the body or colony by avoidance behaviour and consolidated physiological defences in the epithelia or at nest entrances. **2.** Soma defence: immune defence acts to prevent infection of somatic tissue (cells in the body and sterile workers in insect colonies). **3.** Germ line and offspring defence: the reproductive tissue (ovaries) of organisms and the reproductive individuals (queens) of insect colonies are subject to specific immune privileges to prevent offspring infection (taken from Cremer & Sixt, 2009).

2. Termites as superorganisms

Eusociality, interestingly, has evolved multiple times in nature (Wilson & Hölldobler, 2005), and independently so within Isoptera. Isoptera forms a relatively small order of eusocial insects that comprises seven families, which include 281 genera and about 2,600 species (Engel & Krishna, 2004; Kambhampati & Eggleton, 2000). Among eusocial insects, termites are probably the most peculiar as well as phylogenetically oldest eusocial group. Termites, the "eusocial cockroaches", are members of the order Isoptera (Cranston & Gullan, 2009; Inward et al., 2007a; Lewis, 2009). Recent phylogenetic analyses confirmed that termites evolved from subsocial wood-feeding cockroaches and form a sister group with the cockroach genus *Cryptocercus* (Engel et al., 2009; Inward et al., 2007a).

From an ecological perspective, termites are dominant and perhaps vital as decomposers and soil engineers in tropical ecosystems (Eggleton et al., 1996; Inward et al., 2007b). However, as termites feed on cellulose, some species are also renowned for being highly destructive pests of agricultural and silvicultural as well as man-made structures (Abe & Higashi, 2001; Khan & Ahmad, 2018). Thus, the remarkable ecological impact of termites, together with their uniquely social structures and cooperative behaviour (Inward et al., 2007b), makes them ideal model organisms for studying host–pathogen interactions, social evolution, and animal immunity since they are the only insects to have developed eusociality outside the order Hymenoptera. Unlike Hymenopterans, which have the more common holometabolous metamorphosis and a haplo-diploid genetic system, termites are unique among eusocial insects because they are diplo-diploid and have a hemimetabolous mode of development as well as display a diversified caste polyphenism (Korb & Hartfelder, 2008).

Furthermore, termites harbour the most elaborate gut microbiota of any insects. Based on this they can be categorized as higher or lower termites (Engel & Moran, 2013). Although all members of the Termitidae are known as higher termites – characterized by the absence of symbiotic flagellates (Protozoa) in the gut – it is the most diverse termite family, comprising 75% of all termite species. The family Rhinotermitidae is a member of the so-called "lower termites", which are characterized by the presence of symbiotic flagellates along with many species of bacteria in the gut. It contains the majority of the pest species (Abe & Higashi, 2001; Evans et al., 2013; Khan & Ahmad, 2018). The subterranean termites *Reticulitermes flavipes* and *Coptotermes formosanus* (Rhinotermitidae) are species of considerable economic importance (Chouvenc et al., 2008; Su & Scheffrahn, 1998). *R. flavipes* is used as the eusocial insect model in this thesis. This species is known for its ability to excavate complex tunnels (Figure 2) and galleries in the soil, close to decaying wood (Khan & Ahmad, 2018). It is the most broadly distributed termite species in North America (Austin et al., 2005), but has also been reported as invasive in Austria, Germany, France, Italy, Bahamas, Uruguay, and Chile (Evans et al., 2013).

While individual colony members exhibit combined efforts to promote colony productivity and survival, a single termite cannot be considered a normal solitary insect, since it cannot survive or reproduce independently if separated from the colony. This is because the colony functions as an organism-like entity where each caste lacks the ability to perform at least one activity essential for survival, therefore favouring an obligatory association that is otherwise not present in solitary insects (Eggleton, 2010). Colonies of termites are comprised of three major castes: reproductives (queens, kings, and alates) – the sole reproducing individuals; workers and soldiers – the latter two are the sterile individuals that perform specialized tasks. The reproductives are further divided into primary reproductives, which are winged alates prepared to leave the nest to congregate, pair, and start new colonies; and secondary reproductives serving as replacement for dead primary reproductives and responsible for the breeding without dispersal (Korb & Hartfelder, 2008). Once egg production starts, the reproductive castes generally live cloistered in the least accessible and safest part of the nest and cannot feed and defend themselves effectively. Workers, on the other hand, are the most abundant caste. As the workforce of the colony, they are primarily responsible for tasks that allow the colony to function efficiently: they forage for food and feed others, build and repair colony structures, care for the brood and reproductives and also participate in colony defence. Soldiers are specialized at defending the colony due to their powerful mandibles, but they are not capable of feeding themselves. Together, all these functions – reproduction, feeding, defence, and dispersion – are included by necessity in solitary species (Eggleton, 2010).

Figure 2. The eusocial model organism, *Reticulitermes flavipes* in the mini-colony. Two different castes are highlighted with a circle: R: reproductive and W: worker. Clusters of eggs are also indicated (E).

3. Pathogen pressure shapes defence mechanisms: the individual and social immune system of termites

Eusociality has generally been thought to be associated with many costs, including an apparently high susceptibility to disease. Termites represent an interesting case, as they live in particularly dense populations of genetically related individuals that interact intimately likewise in a microbe-rich environment, making them especially vulnerable to infection and rapid disease transmission within the colony (Rosengaus et al., 2010; Schmid-Hempel, 1995; Schmid-Hempel, 2017). This poses a rich scenario for diverse host–pathogen strategies. Clearly, pathogens represent a major selective pressure for termites as well as for other eusocial insects (Liu et al., 2019b; Rosengaus et al., 2010; Schmid-Hempel, 1998). Hosts and pathogens interact in a complex way, resulting in so-called evolutionary arms races, whereby hosts are under pressure to evolve potent defence mechanisms to detect, avoid and exclude pathogens, whereas pathogens in turn adapt by developing strategies to evade or resist host defences and to achieve a successful infection (Baverstock et al., 2009; Dawkins & Krebs, 1979; Liehl et al., 2006). Accordingly, since in termites immune defence is an important life-history trait (Calleri et al., 2010), they have evolved a sophisticated immune system that protects from disease by eliminating pathogens, allowing them to become highly successful. The termite immune system comprises two levels: At the individual level (Figure 3), termites fight off pathogens through effective innate physiological and biochemical defences such as cellular responses and humoral production of effector molecules (Avulova & Rosengaus, 2011; Bulmer et al., 2009; Chouvenc et al., 2009b; Hamilton & Bulmer, 2012; He et al., 2021). At the colony level (Figure 4), termites employ collective disease defences or social immunity to defend against pathogens and alter the success of infection (Bulmer et al., 2019; Davis et al., 2018; Rosengaus et al., 1999a; Rosengaus & Traniello, 2001; Rosengaus et al., 2010; Traniello et al., 2002). The interaction between colony-level and individual defence mechanisms plays a key

role in minimizing pathogen transmission within termite colonies (Fefferman et al., 2007; Lopes et al., 2017).

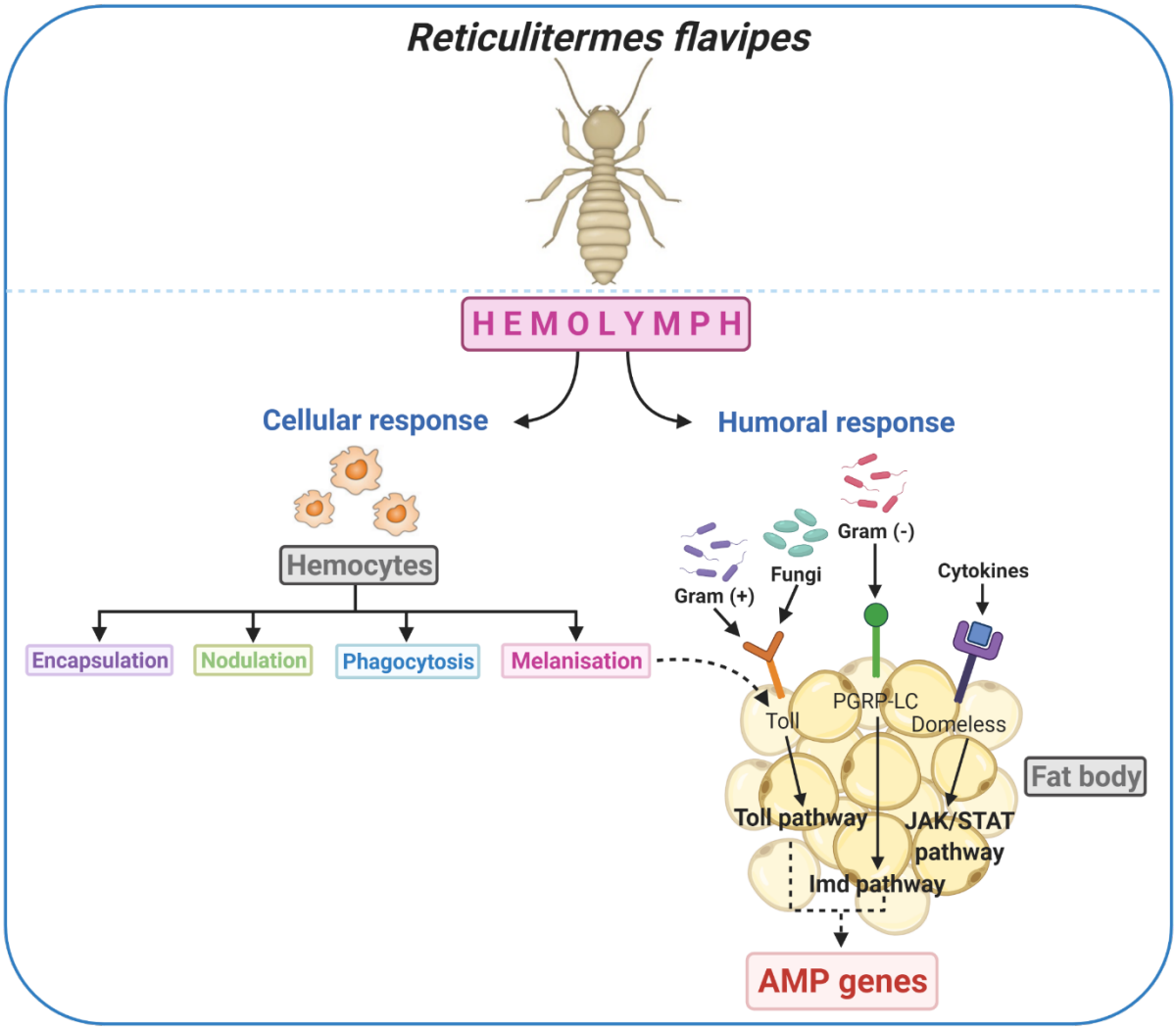

Figure 3. Simplified schematic representation of the *R. flavipes* innate immune response.
The defence consists of the cellular (hemocyte mediated) response (left), as well as the humoral response (right) through the production of AMPs and the melanisation cascade.

3.1. Innate immune system

Since animals are permanently at risk of pathogen infection, their immune systems are essential for their survival. Animals engage diverse mechanisms that discriminate between self and non-self, respond to tissue damage, detain and eliminate non-self, and heal damaged tissues. Unlikely vertebrates, which have evolved a highly specific adaptive immune system, insects rely on an evolutionarily conserved immune system that consists of multiple innate defence mechanisms to detect and combat infection (Babayan & Schneider, 2012). These mechanisms serve as the last line of defence against pathogens and are divided into strictly coordinated cellular and humoral responses (Armitage & Siva-Jothy, 2005; Otti et al., 2014). The latter begin in the hemolymph and comprise three major immune signalling pathways, two of which are regulated by innate immune receptors (Toll and immune deficiency (Imd) pathways) with the third mainly associated with stress factors, tissue damage, or viral

response via hemocyte-released cytokines (Janus kinase/signal transducer and activators of transcription (JAK/STAT) pathway) as well as the melanisation process (Hillyer, 2016; Lemaitre & Hoffmann, 2007). These array of innate signalling pathways are sufficient and mandatory for eradicating most infections (Stokes et al., 2015). Cellular responses, on the other hand, rely on highly differentiated immune cells – hemocytes – which can activate cell-mediated reactions, including phagocytosis of invading organisms by single hemocytes, and multicellular encapsulation and nodule formation (Figure 3) (Hillyer, 2016; Lavine & Strand, 2002; Vilcinskas & Götz, 1999). More specifically, within the cellular innate functions, in phagocytosis, plasmatocytes or granulocytes engulf and destroy a variety of small targets (e.g. bacteria, yeast, double-stranded RNA); in encapsulation, lamellocytes form a multi-layered capsule around larger invaders (e.g. parasites, protozoa, and nematodes); and in nodulation, multiple hemocytes can aggregate and entrap bacteria (Lavine & Strand, 2002; Strand, 2008).

During an infection, the innate immune system recognizes the pathogenic microorganism. This usually occurs when conserved pathogen-associated molecular patterns (PAMPs) such as bacterial peptidoglycans or lipopolysaccharides (LPS), as well as fungal β-1,3-glucans, bind to host-derived pattern recognition receptors (PRRs), initiating the humoral response upon recognition. Furthermore, pathogen recognition is achieved through peptidoglycan recognition proteins (PGRPs) and Gram-negative binding proteins (GNBPs) that trigger the activation of the two primary signalling pathways, Toll and Imd. These pathways induce the expression of genes encoding a battery of broad-spectrum antimicrobial peptides (AMPs) in the fat body and hemocytes, the two mainstay components of insect immunity (Bulmer, 2010; Hillyer, 2016; Stokes et al., 2015). These are subsequently secreted into the hemolymph, helping the insect resist infection (Lu & St. Leger, 2016). The Toll pathway mainly detects and responds to fungal and Gram-positive bacterial infections and mediates, to a large extent, the expression of AMPs through, transduction factors, including *spirit*, which acts extracellularly and upstream of Spaetzle (Kambris et al., 2006), and the NF-κB proteins Dorsal and Dorsal-related immunity factor (DIF). In contrast, the Imd pathway is essential for recognition and response to Gram-negative bacterial infections as well as for controlling different AMP genes via the activation of the NF-κB transcription factor Relish (Lemaitre & Hoffmann, 2007).

Termites, as well as other animals, are by no mean defenceless against pathogens and disease attacks. To fight infections, they use a highly efficient suite of cellular and humoral immune defence mechanisms. In terms of cellular defence, activation of encapsulation and phagocytosis processes against fungal (Avulova & Rosengaus, 2011; Chouvenc et al., 2009b, 2011c) and bacterial infections (Luo et al., 2022) were described. In the case of fungal infection, the number of hemocytes appear to be affected by the progression of disease, specifically when hyphal bodies or blastospores emerge in the hemolymph (Avulova & Rosengaus, 2011). This leads to the hypothesis that termites as well as other eusocial insects rely more on other mechanisms to reduce the risk of infection (López-Uribe et al., 2016). Furthermore, many immune-related genes such as AMPs and pathogen recognition proteins (PRPs) are functionally well-characterized in several insect species, but eusocial insects may

possess a unique humoral immunity against pathogens (Fischman et al., 2011; Wilson-Rich et al., 2009). In termites, recent genomic studies detected a full repertoire of immune genes that are involved in the Toll, Imd, c-jun N-terminal kinase (JNK), and other pathways, suggesting that they may also possess full immune-related pathways present in *Drosophila melanogaster* (He et al., 2021; Hussain et al., 2013; Luo et al., 2022; Terrapon et al., 2014). Four prominent AMPs –spinigerin and prolixicin both antifungal and antibacterial proteins, a termite-unique termicin (a defensin-like gene); and a Gram-negative bacteria binding protein 2 (GNBP-2) – latter two antifungal proteins – were identified in termite salivary glands and hemocytes (Bulmer et al., 2010; Bulmer, 2010; Bulmer et al., 2009; Hussain et al., 2013; Lamberty et al., 2001). Interestingly, termite GNBP-2 has a dual function, first as a PRRs via the sensing of β-1,3-glucans, and second as an antifungal effector due to the robust β-1,3-glucanase activity that breaks down fungal cell-wall components (Bulmer et al., 2009). The β-1,3-glucanase activity of termite GNBP-2 was detected on the termite cuticle as well as in their nest-construction materials, where it operates as a nest-embedded sensor for termite defence as it can signal the presence of fungal entomopathogens prior to cuticular penetration as well as damage and kill them, thereby priming the termite immune system (Bulmer et al., 2010; Bulmer et al., 2009). The detection of the termite GNBP-2 ascribed it a novel fungicidal function. Its efficacy appears to depend on a synergistic interaction with termicins, whereby the β-1,3-glucanase activity of GNBP-2 disrupts the fugal cell wall, allowing termicins to access and interfere with the cell membrane's integrity or to destroy the cell by other mechanisms (Bulmer et al., 2012; Bulmer et al., 2009; Lamberty et al., 2001). Thus, GNBP-2 and termicins appear to provide multifaceted protection not only as internal effectors and receptors but also as external effectors and sensors (Hamilton & Bulmer, 2012; Rosengaus et al., 2010).

In addition to the roles of antifungal defence, an important transcription factor, Relish, homologous to *Drosophila's* Relish (Begun & Whitley, 2000), shows positive selection in termites (Bulmer & Crozier, 2006; Luo et al., 2022), and its activation results in the production of antibacterial peptides (Dushay et al., 1996; Lemaitre & Hoffmann, 2007). Melanisation-related genes, including CTL, serine proteases, and serpin are upregulated in termites and may participate in resistance against bacterial infections (Luo et al., 2022). It is well known that the immune systems are often at the forefront of the ongoing evolutionary arms race with pathogens, leading to strong selective pressure on immune-related genes (Fischman et al., 2011). Both PRPs and AMPs appear to be undergoing positive selection, suggesting a robust arms race between termites and pathogens (Bulmer et al., 2010; Bulmer & Crozier, 2004; Fefferman & Traniello, 2009). Interestingly, immune effectors have been implicated in the evolution of social immunity, indicating that the transition to termite eusociality has led to a reshaping of the immune system towards colony rather than individual defence (He et al., 2021).

3.2. Social immune system

In the evolutionary arms race between termites and their pathogens, innate immune responses are not the sole defence mechanisms. Termites have also developed a type of social immunity whereby individuals mitigate each other's susceptibility to infection. Indeed, social immunity – the collective defences – is frequently the most efficient way to combat pathogens (Cremer, 2019; Cremer et al., 2007; Cremer et al., 2018), and it was proposed to be complementary to the individual immune system (Conroy & Holman, 2021; Van Meyel et al., 2018). In contrast to the highly energetically and metabolically costly innate immunity (López-Uribe et al., 2016; Schmid-Hempel, 2003), social immunity employs less expensive forms of protection against pathogens, ranging from physiological to behavioural defences (Cremer et al., 2007; Rosengaus et al., 2010). Across termites, these defences involve pathogen alarm behaviour, avoidance, meticulous allogrooming one another with the use of antimicrobial secretions, and hygienic behaviours, including cannibalism and burial (Bulmer et al., 2009, 2019; Davis et al., 2018; Rosengaus et al., 1999a, 1999b, 2000; Rosengaus & Traniello, 2001; Traniello et al., 2002; Yanagawa & Shimizu, 2007). All these important strategies impede the entrance, establishment, and transmission of pathogens in the colony (Figure 4) (Cremer et al., 2007). Indeed, such complex collective defence mechanisms displayed by termites and other eusocial insects contribute significantly to the protection against infection at the colony level (Chouvenc & Su, 2012; Cremer et al., 2007; Davis et al., 2018; Pull et al., 2018). Overall, it is believed that the presence of pathogens has heavily influenced the evolution of social immunity and that the defensive success of termites against pathogens lies in these alternative strategies to innate immunity.

Infection starts with a pathogen reaching a susceptible host and interfering with its defences. Thus, hosts may gain selective advantage if they can detect the pathogen and avoid direct contact with it. Avoidance is the first line of defence because it reduces the likelihood of infection by minimising exposure (Baverstock et al., 2009; De Roode & Lefèvre, 2012). Several studies demonstrated that termites prevent hazardous contact by avoiding contaminated areas or nestmates infected with a lethal pathogen (Epsky & Capinera, 1988; Kramm et al., 1982). Avoidance behaviour is mediated either through the detection of olfactory cues (Yanagawa et al., 2010) or communication of information about the presence of pathogenic threats using vibratory alarm behaviour to warn nestmates (Bulmer et al., 2019; Myles, 2002; Rosengaus et al., 1999a). However, the so-called pathogen alarm behaviour is also associated with the aggregation of termites, and increased allogrooming (Bulmer et al., 2019). Allogrooming is considered an inducible defence strategy (Fefferman & Traniello, 2009) that can be activated and stimulated through vibratory signals, as observed in the dampwood termite *Z. angusticollis* when they encounter a pathogen (Rosengaus et al., 1999a).

Commonly, infected termites or even pathogen-odour exposed termites trigger intensive allogrooming by healthy nestmates (Kramm et al., 1982; Rosengaus et al., 1998a; Yanagawa et al., 2011a, 2011b, 2012). Allogrooming functions as the primary component of sanitary care and can boost disease resistance in at least three ways. First, it is an effective way to actively remove pathogens from the insect cuticle to limit the contagion, even after infectious conidia

become firmly attached to the cuticle and start to germinate but before they can penetrate the cuticle and cause an internal infection (Davis et al., 2018). It was also observed that termites preferentially remove more-virulent than less-virulent pathogens by allogrooming (Yanagawa et al., 2012), resulting in low mortality (Liu et al., 2019a). The more virulent the pathogen, the stronger the odour, and the more allogrooming is displayed by termites (Yanagawa et al., 2012). In addition to the reduction of cuticular pathogen loads (more than 80% of infectious conidia) and exposure risk, allogrooming affects pathogen survivorship (Rosengaus et al., 1998a; Yanagawa et al., 2011a; Yanagawa & Shimizu, 2007), as the ability of infectious conidia to germinate is extremely inhibited upon ingestion and exposure to the antifungal activity of the termite's gut (Chouvenc & Su, 2010; Chouvenc et al., 2009a; Yanagawa & Shimizu, 2007).

Second, allogrooming is often accompanied by the secretion and spread of antimicrobial substances over the cuticle and among nestmates to inactivate pathogens and thus reduce infection probability (Bulmer et al., 2010; Bulmer et al., 2009; Hamilton et al., 2011; Lamberty et al., 2001). This suggests that the secretion of antimicrobial substances may be increased during infection. Therefore, antimicrobial secretions appear to be important for disease resistance, given that termicins, GNBP-2, and the β-1,3-glucanase may target pathogens before they penetrate the cuticle, making these secretions effective at protecting termites from internal infection (Bulmer et al., 2010, 2009; Hamilton et al., 2011). Overall, the externalization of antimicrobial secretions illustrates how behavioural and physiological defences may interplay. This could be an important component of social immunity (Rosengaus et al., 2010) and may have been important in overwhelming the high pathogenic pressures associated with eusociality (Hamilton et al., 2011).

And finally, allogrooming can not only be beneficial for the exposed-individual but also for the nestmates, which sometimes can receive a protective "active immunization" by social contact to fungus infected individuals, building up resistance in healthy nestmates. This strategy, characterized by social transfer of low-dose pathogens, allows nestmates to experience infections that are just sublethal by actively upregulating their immune system as observed in termite colonies. Such "active immunization" also primes the immune system of healthy termites, reducing susceptibility to infection (Liu et al., 2015; Traniello et al., 2002).

If these first initial defences fail to prevent infection, and the pathogen nevertheless happens to penetrate the insect cuticle, an internal lethal infection arises and termites fall sick (Chouvenc et al., 2009b; Davis et al., 2018) due to rapid proliferation and toxin secretion of the pathogen inside the host hemolymph (Chouvenc et al., 2009a; Hänel, 1982; Syazwan et al., 2021). Internal pathogens are therefore inaccessible to allogrooming. At this stage, infected individuals may turn into new infection sources inside the colony if the pathogen replicates and produces new infectious conidia. Therefore, the expression of defence mechanisms that limit pathogen replication and transmission among colony members and thus render the whole colony resistant to the disease are required (Liu et al., 2019b).

Accordingly, cannibalism, an adaptative characteristic behaviour exhibited by termites (Richardson et al., 2010), is extensively relied on. The cannibalistic strategy focuses on the "elimination" of infected individuals, just like immune cells destroy infected cells in a body (Cremer, 2019). Hence, once an internal infection is successfully established, social immunity switches from "care" to a more extreme and drastic "kill" strategy (Cremer, 2019; Cremer et al., 2018; Cremer & Sixt, 2009), potentially enhancing colony survival. Although the kill-component is a unique feature of social immunity, it is little studied in comparison to the care-component (Pull et al., 2018). In a study in the termite *Z. angusticollis,* cannibalism was found to occur when infected termites were "moribund but not yet dead", and increased chances of cannibalism were towards individuals with higher concentrations of fungal conidia (Rosengaus & Traniello, 2001). Further, Davis et al. (2018) confirmed that in the subterranean termite *R. flavipes*, cannibalism is displayed after infected termites displayed signs of overt sickness, with higher rates of cannibalism occurring close to the termite's death (Davis et al., 2018). Broadly, cannibalism is beneficial for termite colonies because it kills both the infected individual and the fungus inside via antifungal activity of the termite's gut (Chouvenc et al., 2009a, 2010), hence mitigating against spread.

Once an internal infection is advanced and termites die, a considerable risk of disease transmission possibly emerges within the colony. Whether an infected termite cadaver gets eliminated through cannibalism or burial may depend on fungal colonization (before sporulation) and mortality rate. When *M. anisopliae*-infected termites die, cadavers are primarily cannibalized by healthy nestmates (Chouvenc et al., 2011a, 2008; Chouvenc & Su, 2012). However, when mortality exceeds a threshold indicating that infection has become massive, dead termites are predominantly buried (Chouvenc & Su, 2012; López-Riquelme & Fanjul-Moles, 2013). In addition to its function as hygienic behaviour, ingestion of an infected cadaver aids the inhibition of the fungus due to the termite's gut antifungal activity (Chouvenc et al., 2009a, 2010), which implies that cannibalized cadavers cannot be a source for fungal replication and therefore chances of disease outbreaks are reduced. Unlike cannibalism, burial isolates cadavers from other colony members (Sun et al., 2013). Fungus-killed termites are commonly buried or covered with chewed filter paper (Kramm et al., 1982; Myles, 2002; Zoberi, 1995) or sand particles (Chouvenc & Su, 2010, 2012) because they still constitute a risk of contamination if the fungus is able to sporulate from the cadaver (Hänel, 1982), so the whole colony will be at danger. The use of faecal material and chewed paper, wood or soil coated with saliva for burial provides antifungal components, which also prevent further fungal proliferation (Bulmer et al., 2009; Chouvenc et al., 2012a; Chouvenc & Su, 2010; Rosengaus et al., 1998b; Sun et al., 2003). Importantly, these behavioural changes are mediated by chemical signals. A recent study found that termites perform cannibalism by sensing an early death signal, whereas burial of dead individuals is displayed by sensing late death signals (Sun et al., 2017). Largely, all these strategies are likewise important in controlling disease transmission within termite colonies since they diminish the probability of fungal sporulation.

Detection of pathogens and coordination of social immune responses needs precise communication of wide-range disease-related information (Cremer et al., 2018). Information transfer is primarily done through chemicals that are used as signals by nestmates and influence the behavioural defence (Leonhardt et al., 2016; Lopes et al., 2017). Since eusocial insects change their responses according to the pathogen threat (Fefferman & Traniello, 2009), anti-pathogen defences may be triggered by specific signals of the health status, regardless of disease progression. Infected individuals might be actively emitting "sickness signals" to nestmates – signalling for help when contaminated and signalling for elimination when infection advanced – so that the colony effectively undertakes either sanitary care or elimination behaviours (Cremer, 2019). In this regard, detection of infected nestmates has evolved as a protective mechanism against disease transmission. However, the underlying mechanisms of infection recognition and communication in termites are poorly known. Most strikingly, detection of pathogen-specific odours is frequently a key part of the defensive behavioural repertory of termites (Mburu et al., 2009; Yanagawa et al., 2011b). Yanagawa et al. (2011b) reported that fungal-odour-treated termites significantly mediate an increase in allogrooming. Yet, changes in cannibalism or burial behaviours were not found. These findings suggest that allogrooming is probably enhanced by signals before infection, while cannibalism and burial behaviours are induced by signals after infection (Yanagawa et al., 2011b). In line with these results, Davis et al. (2018) found that allogrooming was elicited during early-stage infection, specifically before and after conidia germination, so pathogen factors might be the main signal for sanitary care, whereas cannibalism was triggered by an internal-stage of *M. anisopliae* infection in which death is inevitable and host condition leading to the elimination of the infected individual (Davis et al., 2018). Hence, the termite colony moves from sanitary care through pathogen-triggered intensified allogrooming to cannibalism, reflecting the "care-kill" strategy in an exciting race against the pathogen. This suggests that termites may have a well-adapted social immune system whereby behavioural interactions help to limit disease outbreaks. Behavioural defences, employed by termites will constitute the core part of the investigations performed in this thesis.

Although allogrooming and cannibalism are well-documented social immune responses in termites, enhancing colony health and survival (Davis et al., 2018; Rosengaus et al., 2010; Yanagawa et al., 2011b), the linked regulation factors responsible for the switch from one behaviour to another during the process of fungal infection have not yet been fully described (Davis et al., 2018). Points above demonstrate the complexity of the phenomenon, and from these examples stem new questions: What is the nature of the signal(s) responsible for the behavioural switch during infection? How do termites recognize infected/moribund members of the colony? Is the information of health status broadcast to the colony? Where does this signal come from? Are behavioural changes elicited only by a single signal from the pathogen or multiple signals from the host? These and many more questions are still to be investigated. It is also worth revisiting the most popular strategy against pathogen replication and transmission, namely cannibalism. This can be achieved by avoiding pathogen infection

through the termite cuticle and instead injecting the pathogen into the termite's hemolymph. This method is exploited throughout Chapters I and II.

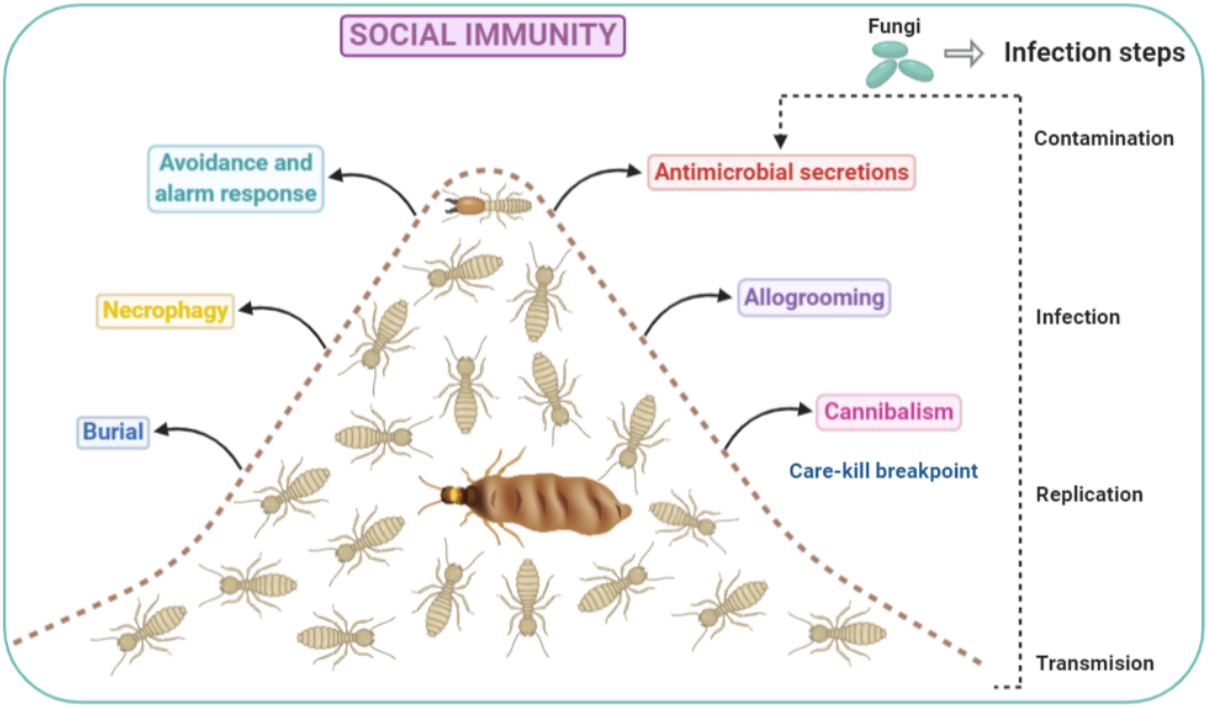

Figure 4. Colony-level immune defences of *R. flavipes* termites against fungal infections.

4. Entomopathogenic fungi: interaction between *Metarhizium anisopliae* and termite hosts

Entomopathogenic fungi with nearly 750 species and 90 genera constitute the most specialized and largest group of natural enemies to umpteen varieties of insect species because they infect and kill insects (Shang et al., 2015; Vega et al., 2012). The majority of the well-characterized entomopathogenic fungi belong to the orders Entomophthorales (Phylum: Entomophthoromycota) and Hypocreales (Phylum: Ascomycota) (Boomsma et al., 2014; Singh et al., 2016; Vega et al., 2012). Entomopathogenic fungi belonging to Hypocreales such as *Beauveria* sp. and *Metarhizium* sp. have shown particularly great promise as biological control agents because unlike other entomopathogens (bacteria and viruses) that usually need to be ingested, they infect their insect hosts directly by penetrating the cuticle to internally proliferate and cause lethal disease (Butt et al., 2016; Pedrini, 2018). Interestingly, the disease caused by *Metarhizium* sp. is known as 'green muscardine', based on the appearance of insect cadavers that are covered with green conidia (Roberts & St. Leger, 2004).

Living in large underground colonies, termites are constantly exposed to a broad variety of different pathogens and are particularly susceptible to those that penetrate the soft termite cuticle (Chouvenc et al., 2013; Milner et al., 1998). Indeed, entomopathogenic fungi are likely to be a serious disease threat to subterranean termites because they are widespread in soil and around termite colonies (Chouvenc et al., 2011b; Dong et al., 2007; Milner et al., 1998; Zimmermann, 2007). *Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota:

Hypocreales), a generalist pathogen that ultimately kills termites after successful infection (Hänel, 1982; Syazwan et al., 2021; Zoberi, 1995), is probably the most well studied microorganism for exploring the mechanisms of host–pathogen interactions, and represents an ideal model entomopathogen to study in the social immunity context (Chouvenc & Su, 2010; Davis et al., 2018; Liu et al., 2019b; Rosengaus et al., 1998a) because it can produce diverse infective propagules, including conidia and unicellular blastospores (Gao et al., 2011; Jackson et al., 2010). Both conidia and blastospores of *M. anisopliae* are highly efficacious in killing individual termites (Chouvenc et al., 2009c; Hänel, 1982; Moino Jr et al., 2002; Neves & Alves, 2004; Sun et al., 2002; Syazwan et al., 2021; Zoberi, 1995). Although blastospores and conidia are both asexual spores, they differ from each other in several ways. The former are thin-walled, pleomorphic, hydrophilic spores produced in nutrient-rich liquid culture and in the insect hemolymph and formed by budding from hyphae. In contrast, conidia have a well-developed cell wall and are uniformily shaped, hydrophobic spores produced on solid substrates or externally on infected insect cadavers (Adámek, 1965; Alkhaibari et al., 2016; Hänel, 1982; Kleespies & Zimmermann, 1992; Sun et al., 2002; Vega et al., 2012). In addition, blastospores are known to germinate more quickly, which is directly correlated with higher virulence (Alkhaibari et al., 2016; Wang et al., 2013). *M. anisopliae* has shown virulence against different termite species (Chouvenc et al., 2009c; Dong et al., 2007) and is known to induce innate immunity in termites, including up-regulation upon infection of defence molecules (Hussain et al., 2013; Rosengaus et al., 2007; Thompson et al., 2003), cellular responses (Avulova & Rosengaus, 2011; Chouvenc et al., 2009b) and collective behaviours (Chouvenc & Su, 2012; Chouvenc et al., 2008; Davis et al., 2018; Myles, 2002; Rosengaus et al., 1998a; Yanagawa & Shimizu, 2007). Therefore, to elicit anti-pathogen defence and to challenge the immune system of our study termite species we used the entomopathogen *M. anisopliae*.

For infection to be successful, fungal entomopathogens must penetrate the insect cuticle, proliferate within the nutrient-rich hemolymph, and avoid the host's immune defences (Butt et al., 2016; Vega et al., 2012). Accordingly, the infection mechanism of *M. anisopliae* against termites (Figure 5), has been well studied: First, the conidia adhere firmly to the thin and poorly sclerotized termite cuticle via specific proteins. Under the right conditions, germination of conidia on the cuticular surface is initiated as early as six hours (Syazwan et al., 2021). After germination, conidia swell, producing germ tubes which differentiate into a specialized infection structure called "appressorium" at 12 hours (Davis et al., 2018; Hänel, 1982), which possibly evolved to overcome host barriers. Penetration into the host is achieved by both mechanical pressure exerted by the appressorium (Butt et al., 2016) and the production of cuticle-degrading enzymes (Schrank & Vainstein, 2010) to allow hyphal penetration and eventually to colonize the insect. A halo around the germ tubes of *M. anisopliae* was associated with the production and excretion of fungal enzymes during the penetration process in the termites *Heterotermes tenuis* (Hagen) (Moino Jr et al., 2002) and *Cornitermes cumulans* (Kollar) (Neves & Alves, 2004). Once the fungus has entered the body cavity, it must overwhelm the innate immune system of the infected host while consuming nutrients for further proliferation. To this end, fungal hyphae grow as yeast-like propagules called hyphal

bodies, analogous to the blastospores, allowing faster and massive multiplication in the hemocoel and the subsequent release of a battery of toxic metabolites, ultimately killing the host (Boomsma et al., 2014; Singh et al., 2016). At this stage of mycosis, termites are observed to show signs of illness (Davis et al., 2018), and moribund termites were always found with dispersed hyphal bodies in the hemocoel (Chouvenc et al., 2009b; Syazwan et al., 2021). This stage has also been attributed to the effect of toxins (Chouvenc et al., 2009a; Gillespie et al., 2000; Hänel, 1982; Syazwan et al., 2021). Kramm & West (1982) found that filtrates of *M. anisopliae* contain toxins that are toxic to termites (Kramm & West, 1982). After the host's death, hyphae emerge from within the cadaver to the outside, releasing new infectious conidia in a process called sporulation. This is the most 'perilous' part of the lifecycle of the entomopathogenic fungus because its persistence and spread through host species relies on this process (Hänel, 1982; Sun et al., 2002; Vega et al., 2012). Commonly, these interactions between entomopathogenic fungi and their insect hosts are known to be antagonistic (Singh et al., 2016) and can be best visualized in Darwin's famous description:

"It follows that any being, if it vary, however, slightly in any manner profitable to itself, under the complex and sometimes varying conditions of life, will have a better chance of surviving, and thus be naturally selected" (Chapter III, Struggle for Existence, Origin of Species, Charles Darwin).

In the ongoing arms race between pathogens and hosts, entomopathogenic fungi have evolved efficient strategies to evade or counter potent immune responses and successfully infect their hosts (Lu & St. Leger, 2016; Qu & Wang, 2018). These strategies are likely to be an important element of the infection process, and they involve, among others, masking the immunogenic components of the fungal cell wall to avoid the activation of immune responses, and secreting immunosuppressive substances to inhibit the immune response in the infected host (Qu & Wang, 2018). *M. anisopliae* is known to synthesize toxic metabolites such as destruxins and other virulence factors during invasion as powerful weapons to suppress the host's cellular and humoral response and facilitate infection (Aw & Hue, 2017; Kershaw et al., 1999; Liu et al., 2019b; Wang et al., 2012). Specifically, destruxins inhibit the expression of AMP genes (Pal et al., 2007) and interrupt hemocyte encapsulation and phagocytosis (Chen et al., 2014; Vey et al., 2002; Vilcinskas et al., 1997). Moribund termites were found to have a decline in hemocyte number (Avulova & Rosengaus, 2011), a lack of structured hemocyte aggregation, or unsuccessful cellular encapsulation due to destruxin production (Chouvenc et al., 2009b, 2011c). This implies that the success of the fungal infection may also depend on the ability of the pathogen to release toxins into the hemolymph before immune recognition occurs (Chouvenc et al., 2009b). Indeed, *M. anisopliae* isolates that produce higher quantities of destruxins are known to be more virulent (Schrank & Vainstein, 2010). In addition to destruxins, *M. anisopliae* can also adopt a camouflage strategy as its blastospores can escape hemocyte recognition by producing a hydrophilic collagenous (Mcl1) protective coat that masks the PAMPs (Wang et al., 2008; Wang & St. Leger, 2006). This protein was detected within 20 minutes of the pathogen contacting the hemolymph (Wang et al., 2008). Although

M. anisopliae can evade the innate immune system once it has entered its host (Wang & St. Leger, 2006), it is unknown to cause natural epizootics in termite colonies (Chouvenc & Su, 2012; Sun et al., 2002, 2003).

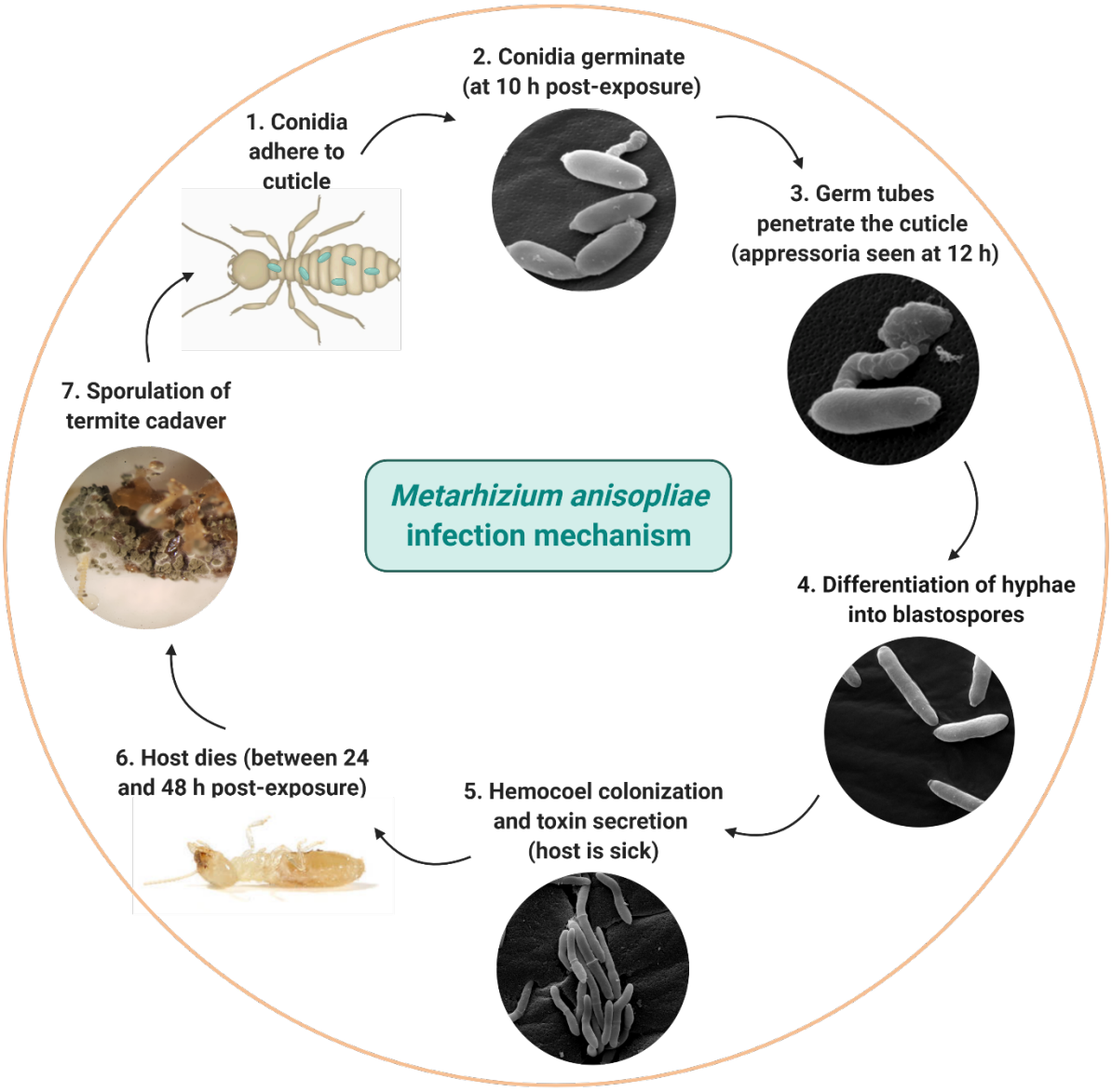

Figure 5. General overview of the invasive and developmental processes of the entomopathogenic fungus *M. anisopliae* in the termite host.
The key infection stages include: **1.** adhesion of infectious conidia to the host cuticle, **2.** differentiation of infection structures (appressoria and penetration pegs) (taken from Davis et al., 2018), **3.** penetration of the cuticle, **4** and **5.** colonization of the hemocoel, **6.** host death, and finally **7.** emergence of the conidiophores for external sporulation on the cadaver.

5. Aim of the thesis

This thesis aims to improve our understanding of how termite societies respond to strong selective pathogenic pressures and how social immunity is achieved effectively to control infectious diseases in two processes. First, whether physiological immune defences are tightly linked with social immune responses. Specifically, we investigated how externally secreted

antifungal molecules derived from the internal immune system regulate collective immune behaviours upon contact with pathogen-exposed individuals. Second, we investigated host–pathogen interactions to understand the mechanism of the care-kill switch of social immunity as well as how specialized social immune responses act against different pathogens, particularly, how a termite colony deals when facing an internal infection and how they detect it. These two processes are important for the survival of the colony, and so these studies lead to a better understanding of the mechanisms that insects have evolved to cope with infectious diseases.

Additionally, we developed a transformation system based on *M. anisopliae* blastospores that consists of the integration of the *bar* and *sur* genes, conferring resistance to glufosinate ammonium (GFS) and chlorimuron ethyl (CME), respectively, and expression of green fluorescent protein (*gfp*) into the fungal genome. The generation of *gfp*-labelled *M. anisopliae* strains offer advantages for better exploring the infection mechanism in termites, specifically during the internal stage of infection, as well as advance understanding of the dynamics of the care–kill dichotomy according to different infection strategies (toxin and growth) from the pathogen. It is also the necessary first step in the development of a genetic toolkit in *M. anisopliae*.

6. Description of project

In this study, two different host–pathogen systems were used to investigate the mechanisms underlying social immunity in a termite colony. The first consists of the model termite species *Reticulitermes flavipes* as a host (Chouvenc & Su, 2010), and *Metarhizium anisopliae* as a fungal entomopathogen (Davis et al., 2018). It will be used to elicit anti-pathogen defences against internal infection. The second consists of the same host species, *R. flavipes*, and the entomopathogenic, Gram-negative bacterium *Pseudomonas entomophila* (He et al., 2021). It will be used to characterize the specificity of anti-pathogen defences. Both pathogens are great models for studying host–pathogen interactions since they can effectively infect a broad range of insects (Liehl et al., 2006; Liu et al., 2019b; Sieksmeyer et al., 2021; Vallet-Gely et al., 2010).

In order to advance our understanding of how pathogens select for social immunity, and, in turn, how social immunity influences the evolution of pathogen virulence, we studied the role of social immunity in termites by facing internal infections either with *M. anisopliae* or *P. entomophila* at different infection stages and levels (varying doses). Specifically, we investigated the underlying mechanisms of the switch from sanitary care, e.g. allogrooming, to elimination behaviours, e.g. cannibalism, which is likely to prevent disease transmission by exploring how elimination behaviours are triggered. We also explored whether these defence mechanisms of the care-kill strategy are pathogen-specific, as well as discover how termites recognize the presence of pathogens and/or diseased nestmates in their colonies. Second, to explore the relationship between physiological immune defences and social immunity in

termites, the suppression of the immune effector molecule Gram-negative binding protein 2 (GNBP-2) and its effect on collective grooming and cannibalism was investigated. Because the regulation of collective immune behaviours may depend in part on the externalized activity of immune effector molecules from the internal immune system.

In **Chapter I** we explored how termites detect and respond to internal infections upon injection of either viable or dead blastospores (within-host infectious form) of *M. anisopliae*. Using a combination of techniques for pathogen injection, behavioural observations, and chemical analysis for detection of termite CHC profiles and *M. anisopliae* destruxins, we elucidated the ability of termites to detect infected individuals at different stages of internal infection and at the same time assessed if pathogen viability might be important for social immunity. Injection of fungal blastospores directly into the hemocoel of individuals allowed us to remove the pathogen as a direct cue that could be used by responding nestmates. We attempted to better study the regulatory factors involved in the care-kill switch and to elucidate the potential identity of the chemical cues that could be responsible for triggering elimination behaviours during the internal infection process.

In **Chapter II** we studied the interaction between *R. flavipes* and *P. entomophila* and elucidated the mechanisms underlying the social immune response of termites against this non-native pathogen, as well as focused on the conditions that trigger specific collective behaviours. Since *P. entomophila* can kill members of *R. flavipes*, we compared termite collective behaviours after injection of *P. entomophila* and *M. anisopliae* blastospores at different infection intensities (viable or dead bacteria, viable or dead blastospores, respectively). We evaluated the sensibility of social immune responses to bacterial versus fungal infections and whether chemical changes are more extensive in fungal than in bacterial infections through detection of cuticular CHC profiles in infected and uninfected termites.

In **Chapter III** we investigated how the inhibition of the antifungal activity of the Gram-negative binding protein 2 (GNBP-2) affects collective immune behaviours in termite colonies upon contact with a pathogen-exposed individual. The experiment, combining behavioural observations and statistical models, sheds light on the link that the antifungal activity of the GNBP-2 has on grooming and cannibalism, and on how these molecules play an important role in the control of infectious diseases in termites. The study presents several novelties: the use of a non-toxic and naturally occurring derivative of glucose – D-d-gluconolactone (GDL) – to block the β-1,3-glucanase activity of GNBP-2 gives opportunities to find new alternatives to biological control and to observe interactions between immune effector molecules and collective behaviours which had not been studied in detail. Furthermore, it serves as the basis for exploration and understanding of how individual immune defences are combining or influence social immunity.

In **Chapter VI** we successfully transformed blastospores from *M. anisopliae* with green fluorescent protein (*gfp*) and resistance to glufosinate ammonium (GFS) and chlorimuron

ethyl (CME) selection markers. Introduction of amplified "*bar-gfp*" and "*sur-gfp*" constructs into competent blastospores was achieved by using the LiAc/ssDNA/PEG method to confer resistance to GFS and CME, respectively. The resistance phenotype was confirmed by transferring mycelia of these colonies to SDNG (synthetic–defined–nitrate–glucose) medium containing the respective selective agent. For genotyping *bar-gfp* and *sur-gfp* (putative) transformants, diagnostic PCR was carried out to detect integration of the amplicons (foreign DNA, or resistance gene linked with *gfp*) into the genome of *M. anisopliae*. The expression of enhanced green fluorescence protein in (putative) transformants was examined for conidia, hyphae, blastospores, as well as on termite cadavers after injection of blastospores within the host hemolymph.

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Chapter I

Altered cuticular chemistry and collective responses following internal fungal infection in the termite *Reticulitermes flavipes*

Altered cuticular chemistry and collective responses following internal fungal infection in the termite *Reticulitermes flavipes*

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Author Contributions

DPM and MAEM conceived the overall idea. MAEM, TL, SJ, LF, VP, RB, and JNT designed the experiments and collected the data. TM and AS performed chemical analysis of fungal destruxins and collected the data. CL, MG, MG and JB performed termite chemistry analysis and collected the data. MAEM, JB and DPM wrote the manuscript. All authors contributed critically to the drafts.

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Abstract

The collective immune defences of advanced social organisms are highly effective at preventing the spread of infectious diseases within their colonies. In insect societies, the "care-kill" paradigm sets out how a compromised individual will either be treated or eliminated from the colony, but little is known about the mechanistic underpinnings of this process. We exploited the stepwise infection dynamics of the entomopathogenic fungus *Metarhizium anisopliae* and its termite host to explore the conditions required for care-kill transition to occur, and the potential identity of the cues that drive these shifts. Due to the costly nature of elimination, we hypothesized that only terminally sick individuals should be removed from colonies. In line with this hypothesis, we found collective responses towards pathogen-injected individuals to vary according to the severity and timing of pathogen challenge, with elimination (via cannibalism) occurring sooner in response to a severe active infection. Unexpectedly, we also found that injection with an equal dose of toxin-depleted inactivated pathogen eventually also resulted in increased elimination rates of compromised individuals, even though this challenge did not necessarily cause host death. This indicates that the threshold at which elimination is triggered occurs at an early stage during the internal host-pathogen interaction cycle; before pathogen viability or even terminal disease status is known. We compared the surface profiles of differently challenged individuals at two time points during the infection process. We found significantly increased amounts of long-chained methyl-branched alkanes in individuals injected with active but not inactive fungal pathogen. This coincided with the highest amounts of observed cannibalism as well as the onset of visible signs of severe moribundity. Together, our findings indicate that termites can detect altered chemical cues on surfaces of infected individuals, which in turn trigger cannibalism. Our study advances our understanding of the influence of pathogens on host chemical cues and highlights the importance of host-pathogen dynamics as drivers of social immunity.

1. Introduction

The evolutionary and ecological success of social insects is widely appreciated. Although sociality offers many benefits, there are also associated costs (Wilson & Hölldobler, 2005). On the one hand, cooperation facilitates division of labour, enabling the optimization of tasks such as brood care, foraging, nest building, information sharing and defence (Aanen, 2018; Cremer et al., 2007; Schmid-Hempel, 2017). On the other hand, densely populated closely related individuals inside a confined nest environment are expected to be exposed to increased risks of disease transmission (Alexander, 1974; Cremer et al., 2018). Pathogens should therefore impose strong selective pressures on their hosts, leading to the potential for the development of collective immune responses, as well as coevolutionary dynamics (Masri & Cremer, 2014). Social insects have evolved effective defensive mechanisms that can protect colonies against disease. Social insect individuals are not only able to fight infections via their own individual immune system (Cotter & Kilner, 2010; De Roode & Lefèvre, 2012; Meunier, 2015), but also via social immune defences (Aanen, 2018; Cremer et al., 2007; Wilson-Rich et al., 2009). Social immunity can be viewed as a distributed organ, similar to the immune system of metazoan animals (Pull & McMahon, 2020) that builds on two principal adaptive pillars – behaviour and physiology – that together aim to prevent the entrance, establishment, and spread of pathogens in a colony (Bulmer et al., 2019; Cremer et al., 2007; Cremer et al., 2018; De Roode & Lefèvre, 2012; Liu et al., 2019a; Schmid-Hempel, 2017).

Social insect groups, such as Hymenoptera (social bees, wasps, and ants) and Isoptera (termites) contain multiple lineages of species with advanced social living, often referred to as superorganisms (Wheeler, 1911). Studies in these social groups have demonstrated the range of collective disease defences that can be deployed against infected individuals (Conroy & Holman, 2021; Cremer et al., 2007; Davis et al., 2018; Myles, 2002; Pull et al., 2018; Wilson-Rich et al., 2007). Infected individuals can be groomed, which in combination with the use of antimicrobial secretions, can serve to cure or rescue the infected individual and reduce the likelihood of internal infection. In contrast, once an internal infection is successfully established, it may pose a threat to the colony and need to be eliminated from the colony (Cremer et al., 2007; Cremer et al., 2018; Cremer & Sixt, 2009; Davis et al., 2018). These different responses towards infected individuals have been described in terms of a “care-kill” dichotomy (Cremer et al., 2018; Cremer & Sixt, 2009; Schmid-Hempel, 1998). Allogrooming is one of the first lines of defence against external pathogens, while elimination typically occurs a later stage (Davis et al., 2018; Esparza-Mora et al., 2020; Pull et al., 2018; Tragust et al., 2013; Ugelvig et al., 2010). Both strategies are thought to reduce the risk of disease outbreak and enhance colony survival (Cremer et al., 2007; Cremer et al., 2018), but the latter imposes an additional cost to the colony through the loss of workforce members.

Collective disease defences are known to vary within and between species and across different levels of social organization (Cremer et al., 2007; Pull & McMahon, 2020; Rosengaus et al., 2010; Ugelvig & Cremer, 2007). Detection of pathogens and the regulation of collective

immune responses depends on the ability of social organisms to accurately communicate disease-related information through behavioural or chemical cues (Cremer et al., 2018; Rosengaus et al., 1999). Chemical communication is thought to play a central role in social immunity (Cremer et al., 2018). For instance, *Lasius neglectus* employs destructive disinfection behaviours, whereby ants initially detect fungus-infected pupae via sickness cues emitted by the pupae, followed by elimination (Pull et al., 2018). Signalling and chemical communication in social insects are often mediated by cuticular hydrocarbons (CHCs) (Hernández López et al., 2017). CHCs are the most abundant class of chemicals coating the cuticle of social insects and have a key role in inter- and intraspecific communication (Blomquist & Bagnères, 2010; J. & D., 2021) potentially also serving to discriminate between healthy and sick nestmates of a colony (Hernández López et al., 2017). CHC profiles can be altered by activation of the immune system (Csata et al., 2017; Hernández López et al., 2017; Richard et al., 2008; Salvy et al., 2001) and altered CHC profiles have been implicated as signals to eliminate infected individuals in ants and honeybees (Baracchi et al., 2012; Hernández López et al., 2017; Pull et al., 2018; Richard et al., 2008) in a manner analogous to body cells signalling their status to the immune system (Cremer, 2019).

Termites also employ several collective mechanisms to prevent disease outbreaks, largely using two complementary behaviours: allogrooming and cannibalism, which reflect the care-kill strategy most typically observed in termites. The switch in the response depends on the progression of the infection, with cannibalism occurring when infected individuals display signs of overt sickness, such as slow movement (Davis et al., 2018). Application of antimicrobial secretions, in addition to behavioural responses, can also effectively remove external pathogens from the cuticle of infected individuals and inhibit pathogen growth during the early-stage of infection (Esparza-Mora et al., 2020; Hamilton & Bulmer, 2012; Kramm et al., 1982; Liu et al., 2015; Rosengaus et al., 1998; Yanagawa et al., 2011; Yanagawa & Shimizu, 2007; Yanagawa et al., 2009, 2010). However, when a pathogen successfully initiates a potentially lethal internal infection (Hänel, 1982; Syazwan et al., 2021), such weak (Chouvenc et al., 2008) or moribund but not yet dead (Rosengaus & Traniello, 2001) individuals are often cannibalized. But the basis of the switch between social immune behaviours in termites remains largely unknown, and it is unclear what signals or chemical cues are employed to trigger elimination behaviours or to what extent they are linked with factors associated with moribundity (Davis et al., 2018).

To address these gaps in our knowledge of social immunity, we investigated how *Reticulitermes flavipes* termites detect and respond to internal infection upon injection of either viable or dead blastospores of the generalist fungal pathogen, *Metarhizium anisopliae*. Blastospores, which represent a within-host infectious form of *M. anisopliae*, play an important role in pathogenesis during disease development and their experimental injection can elicit a robust immune response in insects (Gillespie et al., 2000; Mullen & Goldsworthy, 2006; Vertyporokh et al., 2020; Vilcinskas et al., 1997). Furthermore, blastospores reflect an intermediate stage in the infection process, which begins with conidia adhesion and

germination on the insect cuticle, followed by cuticular penetration via appressoria to colonize the host's body. Within the host, the fungus proliferates as single-celled blastospores or hyphal bodies in the hemolymph ultimately killing the infected insect (Chouvenc et al., 2009b; Chouvenc et al., 2009a; Hänel, 1982; Syazwan et al., 2021). During infection, *M. anisopliae* synthesises virulence factors, such as destruxins (cyclodepsipeptidic mycotoxins) which inactivate the host and assist in clearance avoidance (Gillespie et al., 2000; Iwanicki et al., 2020; Kershaw et al., 1999; Singh et al., 2016; Vilcinskas et al., 1997).

Here, we combine blastospore infection experiments with behavioural observations as well as chemical analyses of termite CHC profiles and *M. anisopliae* destruxins. Our rationale was that to better study the factors regulating the care-kill switch, it is helpful to separate direct external pathogen cues from indirect host-mediated cues, allowing one to investigate how termites detect and respond to internal challenges of varying intensity. Our aim is to shed new light on the care-kill mechanism of the termite *R. flavipes* by investigating how elimination behaviours are triggered by different host immune challenges, and the extent to which these are associated with termite surface profile alterations.

2. Materials and methods

2.1. Termites

The subterranean termite *Reticulitermes flavipes* is characterized by cryptic nesting habits, forming complex colonies whose diffuse nests and multiple feeding sites are connected by underground tunnels (Perdereau et al., 2019; Vargo & Husseneder, 2009). Five *R. flavipes* colonies were used in these experiments: colonies 1, 5, 11+13, E and X. Pieces of wood containing dense aggregations of termites belonging to these colonies were collected from the field. Colonies 5, 11+13 and 1 were collected in Île d'Oléron, France, in 1994, 1999 and 2018 respectively and maintained in a dark room at 26 °C, 84% humidity. Colony E and X were collected in Soulac-sur-Mer, France, in 2015 and maintained in a dark room at 28 °C, 83% humidity. All colonies were kept in separate sheet metal tanks (Becker, 1969), located at the Federal Institute for Materials Research and Testing (Bundesanstalt für Materialforschung und –prüfung) in Berlin, Germany, and had access to wood as well as sufficient damp soil to burrow. Cardboard baits were used to isolate termites from their parental colonies (Dawes-Gromadzki, 2003). Upon isolation, termites from the same colony were transferred to plastic boxes containing cellulose pads (Pall Corporation, Port Washington, USA) that had been moistened with tap water. Collected termites were kept at the same temperature as the parent colony until staining or transfer to experimental Petri dish nests.

2.2. Entomopathogenic fungus

We used the semelparous fungal entomopathogen *Metarhizium anisopliae* (DSM 1490) which has an obligate killing strategy for *R. flavipes* (Chouvenc & Su, 2012; Hänel, 1982; Syazwan et

al., 2021; Zoberi, 1995). *M. anisopliae* induces higher mortality and is well known to trigger a range of behavioural responses in termites (Davis et al., 2018; Syazwan et al., 2021; Yanagawa et al., 2011). Conidia of the entomopathogenic fungus were stored at -70 °C. Prior to each experiment, the conidia were grown on potato dextrose agar (PDA) at 25 °C in darkness until sporulation. After 15 days of incubation the conidia were gently scraped off the plate with a cotton swab moistened with sterile 0.05% Tween 80 and suspended in sterile 0.05% Tween 80. The resulting conidia suspension was vortexed for 30 seconds and then filtered through a piece of sterile miracloth (Merck KGaA, Darmstadt, D) to remove hyphae and large clumps of conidia. The filtered conidia suspension was centrifuged for 10 minutes at 5000 g at 4 °C and the pellet was resuspended and washed three times with sterile 0.05% Tween 80. This protocol was adopted from Davis et al. (2018). A BLAUBRANDR Thoma counting chamber (depth 0.1 mm; BRAND, Wertheim, Germany) was used to estimate the concentration of the conidia suspension. The concentration was adjusted to 1×10^8 conidia/mL with sterile 0.05% Tween 80, aliquoted for ease of use, and stored at 4 °C until further use (within 24 hours). Prior to use, germination rates of conidia were assessed and were over 95% in all cases. To assess germination, we streaked two PDA plates with 50 µL of the 1×10^8 conidia/mL suspension and incubated them in the dark at 25 °C. After 21 hours of incubation, a minimum of 200 conidia per plate were evaluated for germination at 200-400× magnification. Conidia were considered germinated when the elongating germ tube was longer than the maximum conidial diameter (Bernardo et al., 2018).

We injected blastospores in our experiments to enable us to separate the presence of external pathogen cues (conidia exposure) from internal host immune activation (infection initiation). Blastospores are a vegetative cell state that form by budding from hyphae, which develop in the hemolymph of infected insects as a strategy to quickly multiply and colonize the insect body or in nutrient-rich liquid culture (Adámek, 1965; Hänel, 1982; Iwanicki et al., 2020). To prepare cultured blastospores, we added 1 mL of 1×10^8 conidia/mL suspension to 100 mL quantities of two different liquid media in 300 mL Erlenmeyer flasks: (i) 40g/L yeast extract, 80g/L glucose and 0.1% Tween 80; (ii) 40g/L yeast extract, 40g/L glucose, 30g/L corn steep liquor and 0.1% Tween 80 (Adámek, 1965). Erlenmeyer flasks were incubated in a shaking incubator for 3 days (exponential phase) at 25 °C and 290 rpm. Blastospores were then harvested by combining the two cultured media and filtering them through two layers of a sterile miracloth (Merck KGaA, Darmstadt, D) to remove the mycelia. The filtrate was centrifuged for 5 minutes at 2000 g at 4 °C and the pellet containing the blastospores was washed and resuspended three times in Ringer´s ¼ solution. Just as with conidia, blastospore concentrations were determined in a Thoma counting chamber and adjusted to 5×10^8 blastospores/mL. Experimental injections consisted of two pathogen and one control treatment. The two pathogen treatments were: severe challenge, using live blastospores (5×10^8 blastospores/mL viable blastospores; 96% germination rate at 10 hours) or intermediate challenge, using dead blastospores (5×10^8 heat-inactivated blastospores/mL; 0% germination). The control treatment consisted of sterile Ringer´s ¼ solution only. To prepare the dead blastospore treatment, an aliquot of the viable blastospore suspension was

autoclaved. Germination was assessed on two PDA plates streaked with 50 µL of the autoclaved blastospore solution, followed by incubation in the dark at 25 °C for 24 hours. As expected, no fungal growth was observed. Blastospores were considered to have germinated when an elongating germ tube of any size was visible at 200-400× magnification (Bernardo et al., 2018).

2.3. Preparation of experimental colonies

Experimental *R. flavipes* colonies were set up inside Petri dishes as described elsewhere (Davis et al., 2018). Briefly, Petri dishes (94 × 16 mm) were prepared with two cellulose pads (45.5 Ø mm diameter, 0.9 mm thick) (Pall) placed on top of two thin Whatman No. 5 filter paper discs (47 Ø mm diameter, 0.2 mm thick). A standard microscope glass slide (76 × 26 mm) was then placed on top of the filter papers. To establish experimental colonies, we introduced a total of 49 healthy termites (not including the focal individual): 46 medium-to-large workers (3-5 mm body length), 2 representatives of the reproductive caste and 1 soldier. Fifty termites are a functional minimum suggested for lab-based experiments (Becker, 1969) and as described previously, is sufficient to ensure colony establishment, and observe a natural repertoire of termite behaviours (Davis et al., 2018). The paper was moistened with 3.5 mL of tap water prior to the introduction of termites. Experimental nests were sealed with parafilm to maintain a high level of humidity within petri dishes and left in a dark room at 27 °C and 70% humidity for 15 days to enable the termites to establish tunnels under the glass slide. To ensure the clearest possible view into the nest, a cotton swab was used to clean the glass slide of any debris 24 hours prior to the behavioural experiment. The Petri dishes were resealed immediately after cleaning was done.

2.4. Injection of focal termites

We marked focal termites with Nile blue, a moderately toxic fat-soluble stain that has been used in several studies to mark termites and has not been reported to affect termite behaviour (Aguero et al., 2020; Davis et al., 2018). It dyes the termites blue, making them easily distinguishable from their otherwise colourless nestmates. Nile blue dyeing was carried out following a rapid method for marking termites as described previously (Davis et al., 2018; (Evans, 2000). Each focal termite was anaesthetized with CO_2, then injected with 41.4 nL of either viable blastospores, dead blastospores, Ringer´s solution or fungal filtrate (“Filtrate”) directly into the hemocoel using a Nanoject II (Drummond Scientific Company, USA). The needle was inserted into the side of the thorax. With this method, the first stages of the *Metarhizium* infection process that is, adhesion, germination of fungal conidia and cuticle penetration (Butt et al., 2016; Gutierrez et al., 2016) were bypassed. As described above, this allowed us to test the effect of indirect cues triggered by the internal pathogen challenge. By carrying out further experiments with dead blastospore and fungal filtrate treatments, we were able to test whether active infection and terminal sickness were necessary to induce cannibalism.

Injected focal termites were kept individually in small Petri dishes (35 Ø mm), each containing a Pall cellulose pad moistened with 1 mL of distilled water and then incubated for either 2, 8, 12 or 15 hours at 27 °C and 70% humidity post injection (p.i.) before use in behavioural observation experiments. Pathogen dose and incubation times were determined based on blastospore germination and survival assays of termites injected with different concentrations of *M. anisopliae* blastospores (**Supplementary Figures S1, S2**). As expected from previous studies (Alkhaibari et al., 2016; Bernardo et al., 2018; Gillespie et al., 2000), germination and killing rates are faster when using blastospores as compared to conidia (Davis et al., 2018). Representative images and videos of focal termite and nestmate interactions inside experimental colonies were captured with a smartphone (Samsung galaxy S7) and adjusted for brightness and size (Adobe Photoshop [Adobe Systems, San Jose, California]) to highlight different social immune behaviours (Supplementary Videos).

2.5. Experimental design

In our observations, termites injected with the selected dose of viable blastospores were moribund by 16 hours p.i., with death occurring between 22 and 26 hours (**Supplementary Figure S1, Table S1).** Knowledge of the germination and infection timeline of *M. anisopliae* in *R. flavipes* (**Supplementary Figure S1, S2, Table S1**) was used to select four incubation timepoints for behavioural observations experiments: (i) 2 h: blastospores have gained access to the hemocoel but have not yet germinated; (ii) 8 h: blastospores have germinated but the host appears healthy; (iii) 12 h: the host shows first signs of sickness, as indicated by slower movements; and (iv) 15 h: the host is moribund (limited to no mobility). For individuals injected with dead blastospores, symptoms do not appear until 22 h p.i., and only among individuals that subsequently died (**Supplementary Figure S1, Table S1**).

2.6. Blastospore experiment

Focal termites were injected with either a suspension containing 5×10^8 viable blastospores per mL, a suspension containing 5×10^8 dead blastospores per mL, or Ringer´s solution as a control. Following experimental challenge with *M. anisopliae* blastospores, focal termites were kept individually for the pre-assigned incubation times (2, 8, 12 and 15 hours) prior to introduction into experimental plates. For each of the four incubation time points, we conducted 24 replicates from 5 independent colonies for each treatment. Colonies X and E contributed 3 plate replicates per incubation time point and treatment, while colonies 1, 5 and 11+13 each contributed 6. Data for colonies X, E, 5 and 11+13 were collected over 2 experimental repeats (with colony E replacing X in the second repeat) while data for colony 1 were collected from a third experimental repeat. Plates from each incubation time were randomly assigned to one of the three treatment groups after the 15-day colony establishment period had elapsed (Total N=288 plates). Randomisation was achieved by using a random number generator to produce double-digit number combinations, where the first digit corresponded to a colony and the second to one of the treatments.

2.7. Blastospore filtrate experiment

To further narrow down the pathogen components that may be responsible for inducing termite social immunity, we tested the effect of heat-inactivated fungal filtrate (lacking blastospores) on nestmate behaviour. Filtrates consisted of the final Ringer´s ¼ solution used to wash dead blastospores, so should contain subcellular components and metabolites but not larger blastospore-sized structural components, which were removed by filtration. Fifty-four experimental plates of *R. flavipes* from colonies 2, 5, and X (6 plates per colony) and the respective focal termites were randomly assigned to one of the three treatment groups (Ringer, dead blastospores, or fungal filtrate) after the 15-day colony establishment period had elapsed (N=18 per treatment). Focal termites were injected either with a suspension containing dead blastospores, fungal filtrate or Ringer‘s solution, which served as a control. In this experiment, challenged termites were isolated for 12 hours prior to introduction into the experimental colonies. Challenged and control termites were introduced individually into the experimental colonies, which were then resealed with parafilm. The observation period began immediately after the last experimental colony was sealed.

2.8. Behavioural recording

After 2, 8, 12, or 15 hours of incubation, injected focal termites were added individually to the Petri dish nests. To minimize vibrational stimuli that *R. flavipes* are known to be quite sensitive to (Hertel et al., 2011), Petri dishes were not opened or moved after sealing. This process took approximately 10 minutes, and the observation period began immediately after the last dish was sealed. For observations, we adopted a scan sampling method used previously (Davis et al.,2018). For the blastospore experiment, we performed scan sampling of each Petri dish nest every 5 minutes for a total of 3 hours (Altmann, 1974). For the blastospore filtrate experiment, behavioural responses were recorded later, due to the delayed onset of cannibalism in the dead blastospore treatment. Here scan sampling was carried out for 2 hours every 5 minutes at 12, 15, 18 and 21 hours post-injection. Scanning typically took around 1 minute. When necessary, a magnifying glass was used (up to 3x magnification) to better distinguish between observed behaviours and a Samsung S7 smartphone was employed as a digital voice recorder. Observations were performed under constant overhead light at 27 °C and 70% humidity. To minimize observer bias, the experiment was blinded by randomly assigning a treatment to each Petri dish nest prior to the observation period as mentioned in the above section. Focal termites were counted 24 hours after introduction into the groups to quantify survivorship. Behaviours of interest were defined a priori and classified into visually distinguishable and non-overlapping categories relevant to social immunity. We focused on five different behavioural categories (Davis et al., 2018):

Groomed by n (gb): Focal termite is being groomed by N nestmates with no evidence of biting.
Cannibalism (c): Focal termite is being bitten by one or more nestmates and/or focal termite body is no longer intact.

Buried (b): Focal termite has had pieces of paper or faeces placed on top of it. Although increasingly difficult to assess, the termite may still be alive. Note that this behaviour did not get exhibited once during the observation period, which led to it being excluded from the analysis.
Not visible (nv): Focal termite is in a part of the nest where it cannot be observed.
Other (o): Focal termite is alive, intact, and unburied, but nestmates are not interacting with it. This reflects behavioural states unrelated to social immunity.

2.9. Destruxin analysis

Blastospore cultures were prepared as described above, except that Czapek Dox liquid medium was used instead (modified, (Oxoid, England) (Golo et al., 2014). Viable blastospore cultures were subjected to vacuum filtration (Thermo ScientificTM NalgeneTM Rapid-FlowTM, USA) to remove all fungal cells and analysed directly. Dead blastospores were generated as described above. Autoclaved blastospores in Ringer´s solution (as per the dead blastospore treatment) were then subjected to vacuum filtration to remove remaining fungal cells and was used in subsequent the LC-MS analysis. Filtrates were analysed by LC-MS for detection of destruxins. For this purpose, a Synapt G2-S HDMS, equipped with an Acquity UPLC system by Waters Co., Milford, MA, USA, was used. The conditions of the mass spectrometer were optimized for small peptides, using the following settings: (+)-ESI in sensitivity mode; capillary voltage: 3,3 kV, sample cone voltage: 40; source offset: 60; source temperature: 90 °C; desolvation temperature: 250 °C; cone gas flow: 1 L/h; desolvation gas flow: 500 L/h; nebulizer gas flow: 6 bar. Throughout analysis, UPLC grade solvents by Biosolve BV, Valkenswaard, Netherlands. The UHPLC conditions were optimized to an injection volume of 7 µL; flow rate: 0,25 mL/min; eluents: A: water + 0,1% formic acid; B: acetonitrile + 0,1% formic acid; column thermostat: 40 °C; gradient: 0 min: 25% B, 10 min: 40% B, 13 min: 40% B, 15 min: 95% B, 20 min: 95% B, 21 min: 25% B, 25 min: 25% B. This method was adopted following Taibon (Taibon et al., 2014). Tandem mass spectrometric experiments were carried out to confirm respective destruxin structures. Comparison with the MS/MS schemes published in the literature verified the retention times noted in the chromatograms (Jegorov et al., 1998).

2.10. Statistical analysis

2.10.1. Survival of termites kept individually

Statistical analyses were carried out in R version 4.0.2 and RStudio version 1.3.1056.

One hundred and eighty *R. flavipes* termites from three different colonies (11+13, 5 and X) were assigned to one of the following treatments: (i) 60 individuals: Viable blastospores (injected 5×10^8 blastospores/mL); (ii) 60 individuals: Dead blastospores (injected 5×10^8 heat-killed blastospores/mL); (iii) 60 individuals: control (injected Ringer's solution). Survival of each individual was recorded every 12 hours for a total of 240 hours. The effect of treatment

on survival was analysed using Cox proportional hazard models with the package *coxme* (Therneau et al., 2020). Median survival time for each treatment was calculated using the *survminer* package (Kassambara et al., 2017). Control data were right-censored, we uncensored one randomly selected individual from the control treatment per colony, following Tragust et al. (2013). The *glht*-function was used to perform post-hoc pairwise comparisons using Tukey tests with Bonferroni correction, using the package *multcomp* v1.4-10 (Hothorn et al., 2012).

2.10.2. Grooming

The amount of grooming (defined as the number of grooming states/total observed states) in each treatment was analysed by fitting a generalised linear mixed model (GLMM) to the data using the *glmer* function in the R package *lme4* (Bates et al., 2015). A binomial error distribution was used to model proportion data (Crawley, 2014). Models for each incubation time (2, 8, 12 and 15 hours) were composed of treatment and amount of cannibalism (defined as the number of cannibalism states/total observed states) as fixed effects. Petri dish nest ID ("Plate") and colony were used as nested random effects (Plate within colony). The blastospore filtrate models were constructed for each timepoint (12, 15, 18 and 21 hours) and contained treatment, amount of cannibalism and colony as fixed effects and "Plate" as random effect. All models were compared using Akaike information criterion (*AIC*-function in R), which revealed the full model to be the best fit. Model assumptions were checked using the functions *simulateResiduals*, *testDispersion* and *testZeroInflation* contained within the *DHARMa* package (Hartig, 2020). We performed post hoc pairwise comparisons using the *glht* function from the *multcomp* package v1.4-10 with Tukey tests using a Bonferroni correction (Hothorn et al., 2012; Korner-Nievergelt et al., 2015).

2.10.3. Cannibalism

The *survfit*-function from the *survival* package (Therneau et al., 2020) was used to model the onset of cannibalistic behaviour and the *ggsurvplot*-function from the *survminer* package (Kassambara et al., 2017) was used to plot the data. Survival curves were compared using a mixed effects Cox model (*coxme* function from the *coxme* package (Therneau et al., 2020)). For the blastospore experiment, models for each incubation time (2, 8, 12 and 15 hours) included treatment as a fixed effect, and Petri dish nest ID and colony as random effects. For the blastospore filtrate experiment, the model included treatment and colony as fixed effects and "Plate" as a random effect. In the survival curve analysis, all control data was initially right-censored; in order to fit a mixed effects Cox model to the data, it was necessary to uncensor one arbitrarily-selected control replicate from each incubation time (Davis et al., 2018; Tragust et al., 2013). The *glht*-function was used to perform post-hoc pairwise comparisons using Tukey tests with Bonferroni corrections.

2.10.4. Mortality of focal termites at 24 hours after introduction into experimental colonies

Survival of focal termites was assessed 24 hours after introduction into experimental colonies (correspondingly at 26, 32, 36 and 39 hours p.i.). Mortality rates were calculated for each treatment and each timepoint. A Wilcoxon rank sum test was used to perform pairwise comparisons of mortality rate ranks between treatments, using a Bonferroni-correction to account for multiple comparisons.

2.11. Images and scanning electron micrographs (SEMs) of blastospores

Representative SEMs of germinating blastospores on PDA media at different time points (0, 2, 4, 8, 12 and 15 hours (**Supplementary Figure S3**)) were taken to determine the in vitro germination rate. For SEM preparations, small and thin pieces of PDA with blastospores were mixed 1:1 with 25% glutaraldehyde in 0.1 M cacodylate buffer pH 7-7.2 for 60 minutes. After three 5-10 minutes washing steps with 0.1 M cacodylate buffer, the samples were fixed in 1% OsO4 in distilled water for 60 minutes and washed three times in distilled water for 5-10 minutes each. Then the blastospores were transferred into vessels covered with planktonic gauze (mesh-size 10 µm) and were dehydrated in ascending ethanol concentrations (30%, 50%, 70%, 90%, 2 x 100%, 2 x 100% absolutely water free) about 10-15 minutes each. Critical point drying with CO2 was performed with a Balzer CPD 030. The dried samples were transferred onto double-sided adhesive tape on specimen stubs before they were sputtered with gold in a Balzer SCD 040. Images were taken with a FEI Quanta 200 ESEM.

2.12. Cuticular Hydrocarbon analysis

Extractions of pools of three individual termites per treatment were performed in 2 mL glass vials (Agilent Technologies, Santa Clara, California, USA) on an orbital shaker (IKA KS 130 Basic, Staufen, Germany) for 10 minutes. Extracts were subsequently evaporated under a constant stream of gaseous carbon dioxide and then resuspended in 5 µL in a hexane solution containing 7.5 ng/µl dodecane (C12) as an internal standard. Following this, 3 µL of the resuspended extracts were injected in splitless mode with an automatic liquid sampler (ALS) (PAL RSI 120, CTC Analytics AG, Switzerland) into a gas-chromatograph (GC: 7890B) simultaneously coupled to a flame ionization detector (FID: G3440B) and a tandem mass spectrometer (MS/MS: 7010B, all provided by Agilent Technologies, Waldbronn, Germany). The system was equipped with a fused silica column (DB-5MS ultra inert; 30 m x 250 µm x 0.25 µm; Agilent J&W GC columns, Santa Clara, CA, USA) at a temperature of 300 °C with helium used as a carrier gas under a constant flow of 1.8 mL/min. The FID had a temperature of 300 °C and used nitrogen with a 20 mL/min flow rate as make-up gas and hydrogen with a 30 mL/min flow rate as fuel gas. The column was split at an auxiliary electronic pressure control (Aux EPC) module into an additional deactivated fused silica column piece (0.9 m x 250 µm x 0.25 µm) with a flow rate of 0.8 mL/min leading into the FID detector, and another deactivated fused silica column piece (1.33 m x 250 µm x 0.25 µm) at a flow rate of 1.33 mL/min into the mass spectrometer. The column temperature program started at 60 °C and was held for 5 min,

increasing 20 °C/min up to 200 °C and then increasing 3 °C/min to the final temperature of 325 °C, held for 5 min.

CHC peak detection, integration, quantification and identification were all carried out with Quantitative Analysis MassHunter Workstation Software (Version B.09.00 / Build 9.0.647.0, Agilent Technologies, Santa Clara, California, USA). CHCs were identified according to their retention indices, diagnostic ions, and mass spectra as provided by the total ion count (TIC) chromatograms, whereas their quantifications were achieved by the simultaneously obtained FID chromatograms, allowing for the combination of the best-suited method for hydrocarbon quantification (Agilent Technologies, Waldbronn, Germany, pers. comm.) with reliable compound identifications. Absolute CHC quantities (in ng) were obtained by standardizing all peak areas of the identified CHC compounds according to the concentration of the internal C12 standard, and additionally divided by three to obtain CHC amount equivalents per single individual. A discriminant analysis (DA) was performed with the R package “MASS” to test whether CHC profiles statistically differ between the investigated treatment groups and to visualize the degree of separation between them. Wilk's λ was calculated to measure the quality of the DA. Furthermore, quantitative differences between the treatments for each individual CHC compound were accessed with Benjamini-Hochberg corrected Man-Whitney U tests and visualized with boxplots where significant differences were detected.

3. Results

3.1. *In vitro* blastospore germination rate

In vitro M. anisopliae blastospore germination was deemed positive when a germ tube was seen projecting from the blastospore. We used SEM to quantify germination (**Figure 1A, B, C, D**) and observed germination of blastospores on PDA plates as early as 4 hours after inoculation, as evidenced by the formation of germ tubes (**Figure 1B, Supplementary Figure S2**), while the earliest time reported in the literature is 2.7 hours after inoculation of *Isaria fumosorosea* blastospores (Altre & Vandenberg, 2001). At 8 hours, germination and elongation of germ tubes continued and by 12 hours a thick mass of elongated hyphae, forming the mycelium (**Figure 1C, D**) could be observed. Blastospores are known to generally germinate faster than conidia. Our observed rate of in vitro blastospore germination is faster than either *in vitro* or *in vivo* (on cuticle) conidia germination in *R. flavipes* (Davis et al., 2018; Syazwan et al., 2021).

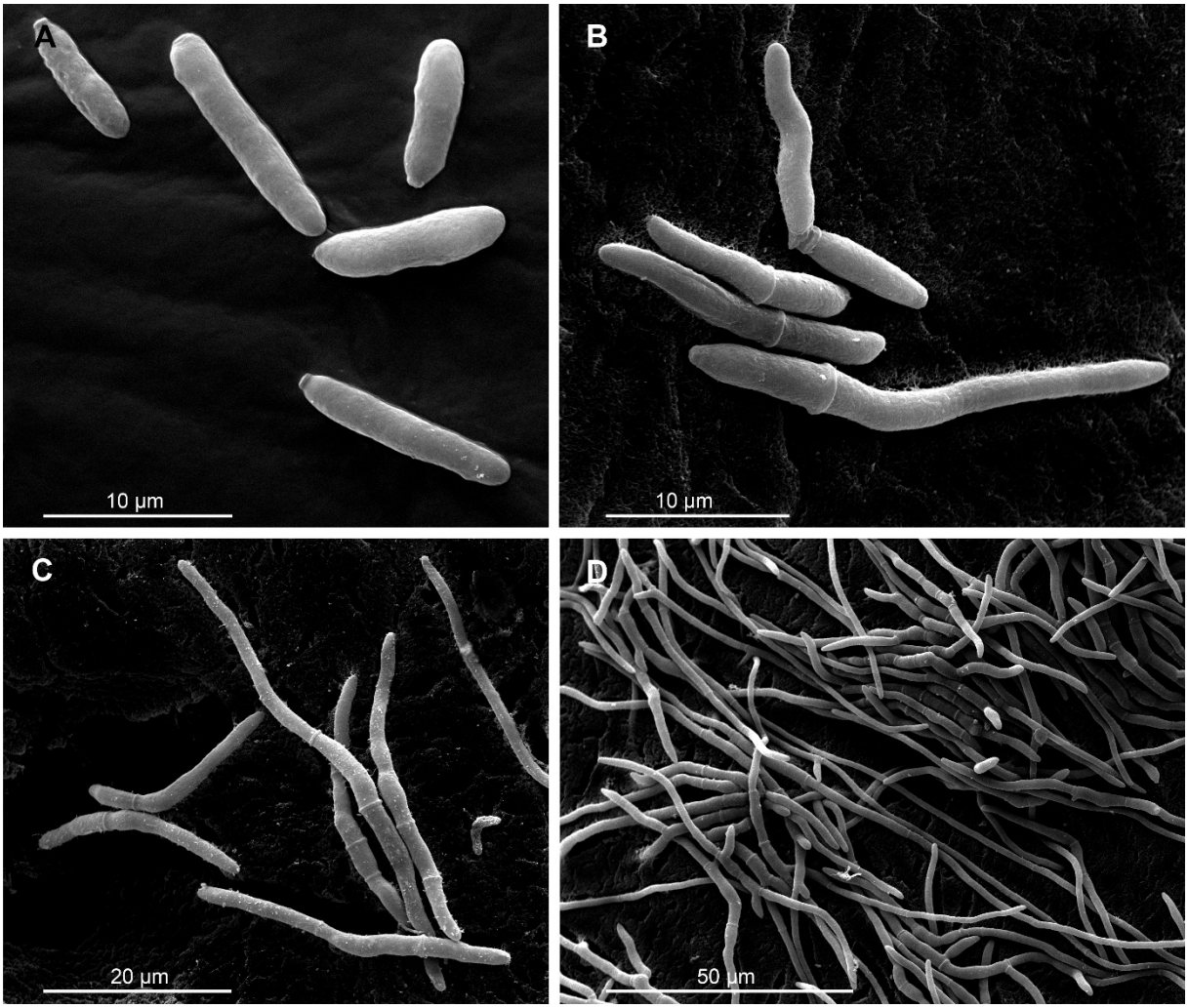

Figure 1. Germination process of *M. anisopliae* blastospores. A-D: Scanning electron micrograph (SEM) images of *M. anisopliae* blastospore germination at different time-points post-inoculation in vitro (PDA media). At 2 h blastospores are not yet germinated (A), while at 4 h, germination of blastospores can already be observed (B), which continues to an advanced stage via germ tube elongation at 8 h (C). By 12 h, elongation of the fungal hypha has continued, and mycelial networks are now visible (D).

3.2. Destruxin analysis

The presence of 5 major classes of destruxins: Destruxin A, B, C, D and Ed was confirmed in the filtered media (CD modified media) of cultured *M. anisopliae* blastospores (**Supplementary Figure S4**). Comparisons with the MS/MS schemes published in the literature verified the retention times observed in the chromatograms (Jegorov et al., 1998). No destruxins were detected in the filtrate derived from heat-inactivated (dead) blastospores (**Supplementary Figure S5**), indicating that destruxins were destroyed by autoclaving.

3.3. Effect of viable blastospore injection on individual termite mortality

Significant differences in survival of termites kept individually were observed between treatments (**Figure 3A**). Survival following injection with dead or viable blastospores were significantly different from Ringer (Blastospores- vs Ringer z = 3.522 *P* = 0.0013; Blastospores+

vs Ringer z = 5.033 *P* = < 0.0001). There was also a significant difference in survival between dead and viable blastospore treatments (Blastospores+ vs Blastospores- z = 8.458 *P* < 0.0001). Viable blastospores caused 100% mortality at 2.5 days post injection, whereas dead blastospores plateaued at 40% mortality by day 3.

3.4. Termites slightly increase allogrooming following internal challenge

Following challenge with Ringer solution or *M. anisopliae* blastospores (dead or viable), injected focal termites were isolated for 2, 8, 12 and 15 hours prior to introduction into observation arenas. Behavioural patterns in both dead and viable blastospore treatments were similar, in that they consisted of low levels of grooming, high amounts of cannibalism, no observations of burial (**Supplementary Figure S6**), as well as "other" states (states unrelated to social immunity). Behavioural patterns in the control treatments were characterized mostly by other states, low levels of grooming and one incident of cannibalism in the 15 hours incubation time. We did not observe burial in any of the treatments. In some cases, focal termites were not visible throughout the three-hour observation period.

The overall proportion of allogrooming in both dead blastospores and viable blastospores treatments were significantly higher than control treatments except for 8h/Blastospores-, 8h/Blastospores+, 15h/Blastospores+ and 15h/Blastospores- **(Figure 2)** (2h/Blastospores- vs 2h/Ringer z = 3.293 *P* = 0.0030; 2h/Blastospores+ vs 2h/Ringer z = 4.448 *P* = < 0.0001; 12h/Blastospores- vs 12h/Ringer z = 4.324 *P* = < 0.0001; 12h/Blastospores+ vs 12h/Ringer z = 4.515 *P* = < 0.0001). The low proportion of allogrooming observed within 15h/Blastospores- and 15h/Blastospores+ treatments is linked with a high proportion of cannibalism (**Figures 2, 3B, Supplementary Table S1**).

In the blastospore filtrate experiment, the overall proportion of allogrooming was at its highest 12 hours post infection and decreased with time for the two fungal treatments (dead blastospores and fungal filtrate) while remaining unchanged for the control group, with no significant differences detected between any of the time points. Allogrooming was significantly elevated in termites treated with dead blastospores over the controls at 12 hours but not at any other stage of infection (**Supplementary Figure S8**). The overall proportion of allogrooming in both dead blastospores and fungal filtrate was significantly higher over control treatments on two occasions (12h/Blastospores- vs Ringer z = 7.636 *P* = 0.0001; 12h/Filtrate vs Ringer z = 5.349 *P* = 0.0001; 15 h/Blastospores- vs Ringer z = 6.626 *P* = 0.0001; 15h/Filtrate vs Ringer z = 4.634 *P* = 0.0001). Allogrooming was significantly different between dead blastospores and fungal filtrate treatments only at 12 hours (12h/Blastospores- vs Filtrate z = -2.510 *P* = 0.0363). A progressively lower level of grooming corresponded with an increasingly higher proportion of cannibalism for the time points 15 and 18 hours and with a high number of termites already being eliminated by 21 hours post infection (**Supplementary Table S4, Figure S8, S9 and S10**).

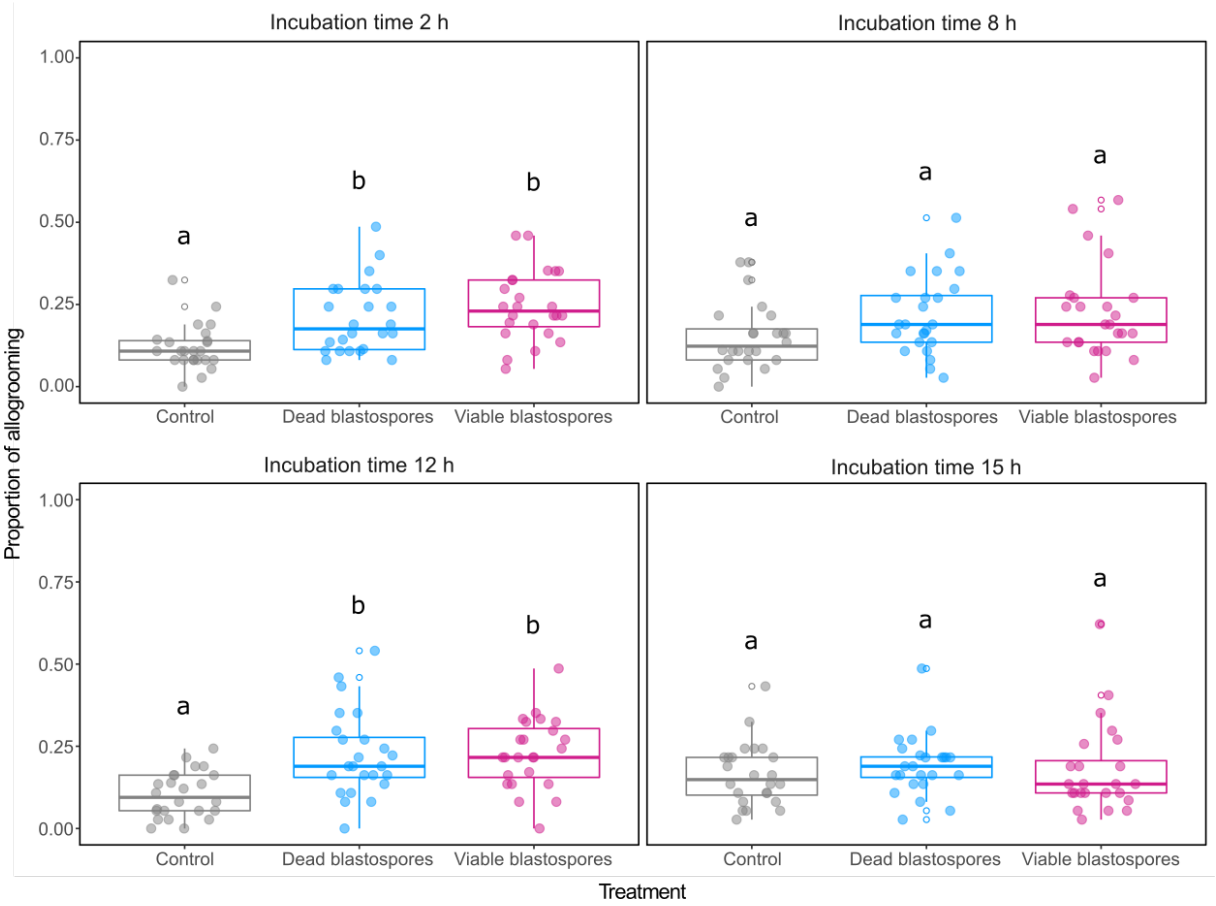

Figure 2. Allogrooming as a proportion of total states across treatments in the blastospore experiment. The proportion of allogrooming states depends significantly on treatment. Significant *post hoc* comparisons are indicated by different letters, derived from separate GLMMs from each time point. Note that *post hoc* comparisons do not apply between incubation time points. Lower and upper hinges correspond to first and third quartiles. Upper and lower whiskers extend to the largest/smallest value if no greater/smaller than 1.5 times the inter-quartile rage from the hinge, respectively.

3.5. Termite moribundity is not necessary for cannibalism

The highest amounts of cannibalism were observed at 15 hours in the viable blastospores treatment, with cannibalism beginning shortly after introduction of the focal termite, and completely replacing grooming before the end of the observation period (**Supplementary Figure S6**). We observed that nestmates cannibalized both dead and viable blastospore injected focal individuals, whereas control focal termites were left unharmed (**Figures 3B**, **Supplementary Table S3, Figure S7**). Cannibalism occurred in both *M. anisopliae* blastospore (dead or viable) treatments, but sooner and more frequently when infected focal termites were visibly ill and close to death (viable blastospores at 15 hours incubation time). At the time of cannibalism, all focal termites were still alive, consistent with the survival of individual termites when kept in isolation following challenge with Ringer's solution, dead or viable blastospores (**Figure 3A**).

Although all the fungal treatments exhibited generally lower survival, the probability of remaining unharmed did not differ between the dead and viable blastospores treatments except at the 15 hours incubation timepoint (15h/Blastospores+ vs Blastospores- z = 4.054 *P* < 0.0002). At 15 hours focal termites infected with viable blastospores treatment were quickly dismembered. A significant difference in the probability of remaining unharmed was found in comparisons of viable blastospore treatments with Ringer-injected controls (2h/Blastospores+ vs Ringer z = 3.312 *P* = 0.0028; 8h/Blastospores+ vs Ringer z = 3.226 *P* = 0.0038; 12h/Blastospores+ vs Ringer z = 2.808 *P* = 0.0150; 15h/Blastospores+ vs Ringer z = 4.822 *P* = < 0.0001). Except for 1 individual in the 15h/Ringer treatment, cannibalism was not observed for any Ringer-injected focal termites (**Figure 3B, Supplementary Table S3**). Cannibalism was observed in all dead-blastospore treatments and comparisons with Ringer-injected controls were also significant (2h/Blastospores- vs Ringer z = 3.114 *P* = 0.0055; 8h/Blastospores- vs Ringer z = 2.397 *P* = 0.0497; 12h/Blastospores- vs Ringer z = 2.946 *P* = 0.0097; 15h/Blastospores- vs Ringer z = 2.684 *P* = 0.0218) (**Figure 3B, Supplementary Table S3**). A large proportion of termites injected with dead blastospores were able to survive when kept individually (approximately 40%, **Figure 3A**). However, when focal termites injected with the same treatment were introduced into experimental colonies, these were consistently cannibalized by 24 hours after introduction (**Figure 3C**). Elimination of individuals at 24 hours was significantly higher for both viable and dead blastospore treatments compared with controls (26h/Blastospores- vs Ringer *P* = 0.0032; 32h/Blastospores- vs Ringer *P* = 0.0044; 36h/Blastospores- vs Ringer *P* = 0.0018; 39h/Blastospores- vs Ringer *P* = 0.0012; 26h/Blastospores+ vs Ringer *P* = 0.0018; 32h/Blastospores+ vs Ringer *P* = 0.0023; 36h/Blastospores+ vs Ringer *P* = 0.0012; 39h/Blastospores+ vs Ringer *P* = 0.0012), with close to 100% of all focal individuals being eliminated by this time point in both viable and dead blastospore treatments. The percentage of eliminated focal individuals at 24 hours did not significantly differ between viable and dead blastospore-injected individuals (26h/Blastospores+ vs Blastospores- *P* = 0.5150; 32h/Blastospores+ vs Blastospores- *P* = 0.2244; 36h/Blastospores+ vs Blastospores- *P* = 1; 39h/Blastospores+ vs Blastospores- *P* = 1).

In the fungal filtrate experiment, significantly increased cannibalism was observed in both dead blastospores and fungal filtrate-injected treatments compared to the control (Blastospores- vs Ringer z = 3.587 *P* = 0.000838; Filtrate vs Ringer z = 3.339 *P* = 0.0021) (**Supplementary Table S5, Figure S10**). Termites challenged with dead blastospores were generally cannibalized at a higher rate, although the amount of cannibalism and therefore overall survival probability was not significantly different between dead blastospore and fungal filtrate injection (**Supplementary Table S5**).

3.6. Cuticular hydrocarbon analysis

A total of 90 individual equivalents of CHC extracts from worker pools (3 individuals each) taken from 3 different *R. flavipes* colonies were pre-defined into six groups for a discriminant analysis (DA) according to the different treatments: 12 and 15 hours after injection of viable

and dead blastospores and Ringer´s solution as a control, respectively (see above). Overall differentiation of the CHC profiles was significant according to the different treatments (Wilk's $\lambda < 0.05$, $P < 0.05$). Discriminant function 1 accounted for 47.36% and discriminant function 2 for 26.11% of the total variation, amounting to 73.47% of total variance explained by the first two functions (**Figure 4**). Concerning the variations in specific CHC compounds according to treatments, no significant differences were detected comparing treatments at 12 hours after injection (**Table 1**). However, after 15 hours of injection, four methyl-branched alkanes consistently displayed significantly higher quantities in viable blastospore versus Ringer's solution treatment ($P < 0.05$, Benjamini-Hochberg corrected Mann-Whitney U tests, **Figure 5**). These compounds were 11-MeC35, 12-MeC36, 11-; 13-MeC37, and 11,15-DiMeC37. Injections with dead blastospores also led to a visible, albeit non-significant increase in overall quantities of these compounds compared to the controls. An overview of all identified CHCs including their respective absolute quantities per treatment is given in **Table 1**.

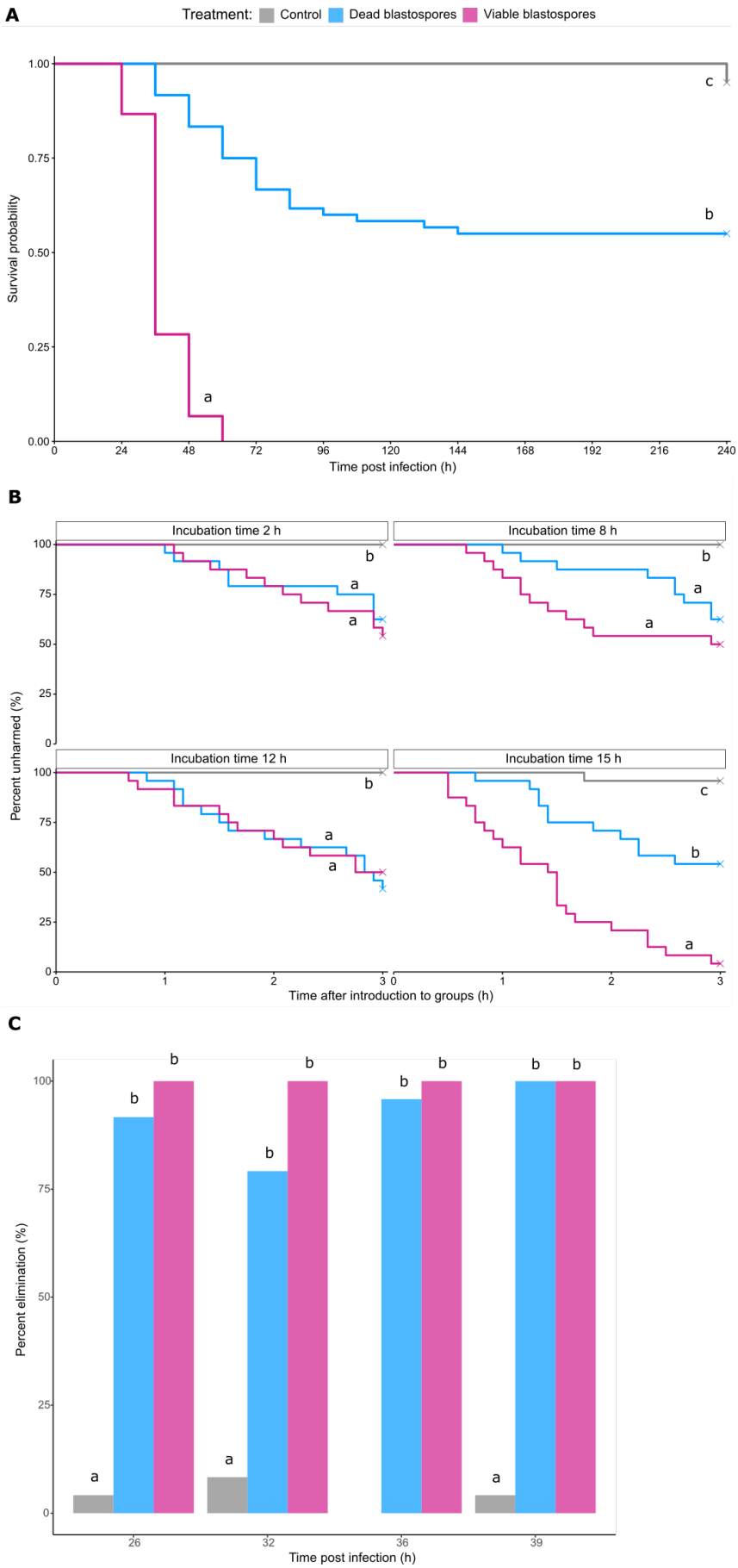

Figure 3. Survival of focal termites kept in isolation (A) and following introduction into experimental colonies (B, C). **A:** Survival of individual termites when kept in isolation. The x axis indicates time post

injection (in hours) while the y axis depicts survival probability, calculated as the proportion of surviving termites from each treatment group. **B:** Percentage of focal termites that remain unharmed (due to cannibalism) after different incubation times (2, 8, 12, 15 h). Cannibalism states were recorded over a three-hour observation period following introduction into experimental colonies. In both survival analyses, crosses indicate the presence of right-censored data (i.e. focal termites that were not dead (A) or harmed (B) during the observation period). Significant differences between treatments derived from survival models are indicated by different letters. Note that *post hoc* comparisons do not apply between incubation time points. **C:** Percentage of focal termites that had been eliminated at 24 h following their introduction into experimental colonies, corresponding to 26, 32, 36 and 39 h post-injection for incubation timepoints 2, 8, 12 and 15 h, respectively. Note that at equivalent timepoints for dead blastospore-injected termites kept in isolation (panel A), mortality was lower than 20%. Treatments: Grey = Ringer (control), Blue = dead blastospores, Pink: viable blastospores.

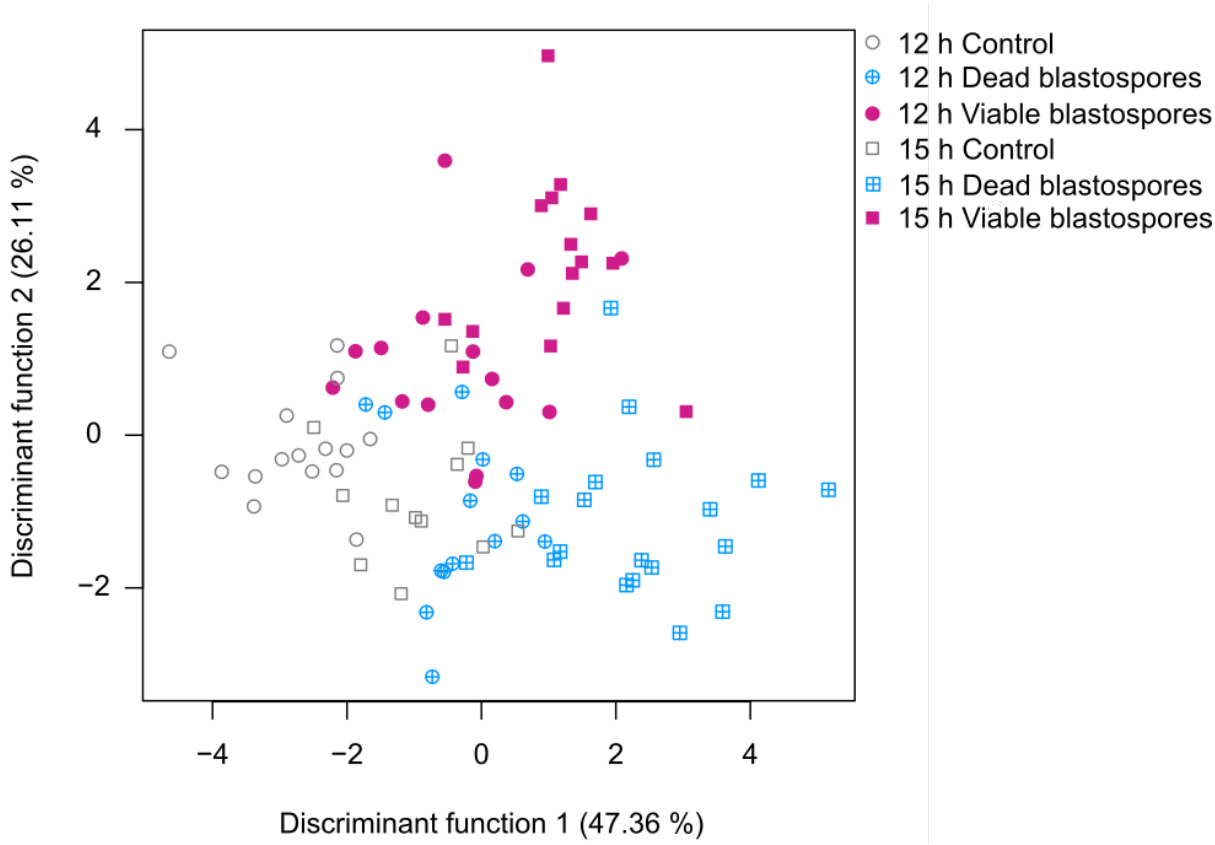

Figure 4. Linear discriminant analysis (LDA) based on 90 cuticular hydrocarbon (CHC) extracts from worker pools (3 individuals each) taken from 3 different *R. flavipes* colonies. The workers were treated with viable and dead blastospores, and Ringer solution as a control, and they were freeze-killed 12 or 15 h after injection. Overall differentiation was significant according to the different treatments (Wilk's $\lambda < 0.05$, $p < 0.05$), the respective contributions of the two discriminant functions to the total variation are indicated in percentages. Different colors and symbols indicate different treatments and time points, the different colony affiliations are not indicated due to them not resulting in consistently discernable differentiation.

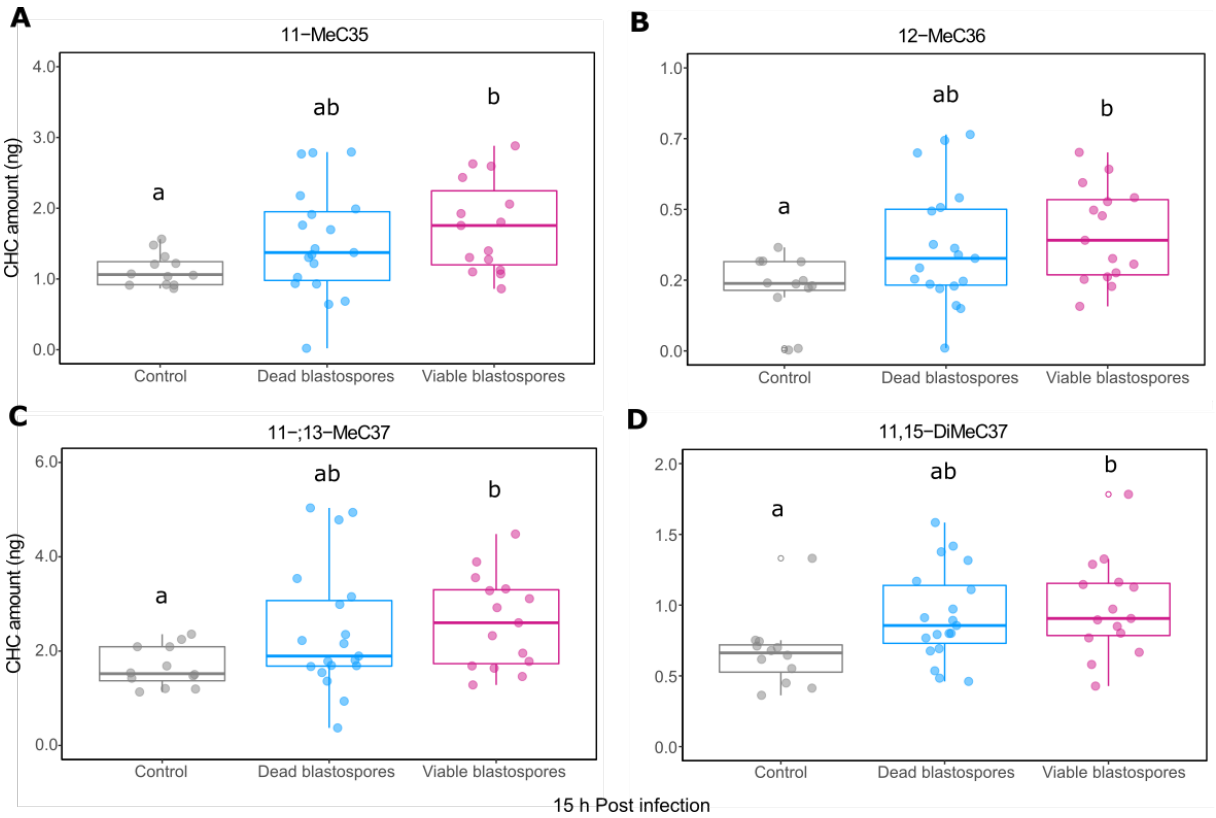

Figure 5. Quantitative comparison of a subset of CHC compounds (three mono- and one di-methyl-branched alkane) 15 h post injection of viable and dead blastospores, or Ringer solution as a control. CHC amounts were measured in ng as individual equivalents from extracts of worker pools (3 individuals each) taken from 3 different *R. flavipes* colonies. The absolute quantities of each of these compounds were significantly increased 15 h after injection of active blastospores as compared to the Ringer control (Benjamini-Hochberg corrected Man-Whitney U tests). Although injection with inactive blastospores did not lead to significant increases after 15 h, an upward trend is observable.

Table 1. Retention indices (RI), compound identifications and the respective absolute quantities as well as standard deviations (in ng) for all CHCs compared between treatments of *R. flavipes* workers: 12 and 15 hours post injection of viable and dead blastospores and Ringer solution as a control, respectively. Retention indices were calculated according to the position of detected *n*-alkanes in our samples and, where not available, with a C21-40 *n*-alkane standard run under the same conditions. Significantly different compound quantities are indicated in bold, accessed through Benjamini-Hochberg corrected Mann-Whitney U tests.

RI	Compound ID	12 h Dead blastospores	12 h Viable blastospores	12 h Control	15 h Dead blastospores	15 h Viable blastospores	15 h Control
2207	n-C22	0.12 ± 0.07	0.11 ± 0.08	0.09 ± 0.08	0.12 ± 0.06	0.15 ± 0.1	0.08 ± 0.05
2270	3-MeC22	0.09 ± 0.26	0.04 ± 0.05	0.02 ± 0.02	0.03 ± 0.02	0.04 ± 0.04	0.1 ± 0.27
2280	C23-ene_1	1.15 ± 0.69	0.96 ± 0.59	0.84 ± 0.69	1.4 ± 0.71	1.64 ± 1.35	0.86 ± 0.66
2287	C23-ene_2	0.07 ± 0.03	0.07 ± 0.04	0.05 ± 0.03	0.09 ± 0.03	0.11 ± 0.06	0.05 ± 0.02
2310	n-C23	9.2 ± 5.76	8.28 ± 5.93	8.04 ± 6.3	9.77 ± 4.21	11.69 ± 7.68	7.34 ± 4.79
2343	11-;13-MeC23	0.77 ± 0.47	0.66 ± 0.41	0.59 ± 0.44	0.92 ± 0.42	1.04 ± 0.72	0.63 ± 0.43
2371	4-MeC23	1.61 ± 1.08	1.54 ± 1.09	1.27 ± 0.87	1.87 ± 0.84	2.21 ± 1.46	1.33 ± 0.84
2380	C24-ene	0.57 ± 0.33	0.53 ± 0.37	0.43 ± 0.3	0.67 ± 0.23	0.82 ± 0.53	0.45 ± 0.29
2409	n-C24	5.66 ± 2.97	5.23 ± 3.11	5.03 ± 2.89	5.93 ± 1.61	6.89 ± 3.6	4.85 ± 2.28

2442	12-;14-MeC24	1.45 ± 0.77	1.36 ± 0.79	1.12 ± 0.68	1.82 ± 0.65	1.96 ± 1	1.27 ± 0.73
2463	C25-ene_1	0.1 ± 0.04	0.12 ± 0.04	0.1 ± 0.05	0.12 ± 0.04	0.16 ± 0.08	0.1 ± 0.03
2473	4-MeC24	16.46 ± 8.66	16.46 ± 9.71	13.92 ± 7.83	18.29 ± 5.39	22.33 ± 11.84	13.99 ± 6.49
2483	C25-ene_2	9.58 ± 5.23	9.19 ± 6.17	7.3 ± 4.86	11.7 ± 4.15	14.79 ± 8.41	7.7 ± 4.07
2483	C25-diene_1	6.02 ± 3.87	5.97 ± 4.53	4.49 ± 2.77	6.97 ± 3.69	8.9 ± 6.29	4.64 ± 3.12
2509	n-C25	25.88 ± 12.72	24.05 ± 13.28	23.74 ± 12.34	27 ± 6.45	31.66 ± 15.7	22.96 ± 9.55
2542	9-;11-;13-;15-MeC25	21.06 ± 11.28	19.47 ± 11.1	16.07 ± 9.25	24.94 ± 7.95	27.99 ± 14.37	18.62 ± 10.35
2555	C25-diene_2	3.29 ± 1.75	3.12 ± 1.81	2.72 ± 2.17	3.59 ± 1.61	4.74 ± 3.51	2.48 ± 1.39
2557	5-MeC25	2.58 ± 1.87	2.14 ± 1.68	1.68 ± 1.16	2.96 ± 1.99	4.09 ± 3.49	2.11 ± 1.52
2575	4-MeC25	3.37 ± 1.59	3.22 ± 1.59	2.7 ± 1.48	3.89 ± 1.05	4.67 ± 2.17	2.78 ± 1.19
2577	C25-diene_3	3.19 ± 1.95	3.15 ± 2.31	2.7 ± 1.5	3.66 ± 1.53	4.37 ± 2.72	2.86 ± 1.63
2581	3-MeC25	6.34 ± 3.15	6.24 ± 3.48	5.41 ± 3.11	7.06 ± 2.21	8.7 ± 4.72	5.59 ± 2.89
2641	12-;14-;16-MeC26	0.69 ± 0.45	0.63 ± 0.44	0.49 ± 0.31	0.73 ± 0.36	0.9 ± 0.51	0.58 ± 0.33
2651	6-MeC26	0.21 ± 0.09	0.2 ± 0.1	0.18 ± 0.12	0.25 ± 0.11	0.34 ± 0.28	0.2 ± 0.12
2669	4-MeC26	0.78 ± 0.42	0.71 ± 0.51	0.65 ± 0.39	0.89 ± 0.34	1.05 ± 0.56	0.69 ± 0.36
2681	3-MeC26 + C27ene	0.15 ± 0.1	0.14 ± 0.08	0.23 ± 0.2	0.18 ± 0.11	0.27 ± 0.17	0.14 ± 0.09
2711	n-C27	0.22 ± 0.11	0.18 ± 0.12	0.47 ± 0.36	0.28 ± 0.37	0.76 ± 2.11	0.28 ± 0.24
2742	11-;13-;15-MeC27	0.11 ± 0.05	0.08 ± 0.05	0.07 ± 0.04	0.12 ± 0.05	0.14 ± 0.07	0.1 ± 0.06
2744	C27diene1	0.29 ± 0.17	0.25 ± 0.17	0.21 ± 0.2	0.29 ± 0.18	0.45 ± 0.4	0.21 ± 0.15
2756	C27diene2	0.11 ± 0.05	0.09 ± 0.07	0.09 ± 0.05	0.13 ± 0.07	0.14 ± 0.06	0.11 ± 0.04
2792	5,17-DiMeC27	0.06 ± 0.03	0.05 ± 0.03	0.05 ± 0.04	0.07 ± 0.04	0.09 ± 0.11	0.05 ± 0.04
3531	**11-MeC35**	1.33 ± 0.62	1.44 ± 0.8	1.07 ± 0.41	1.52 ± 0.76	**1.75 ± 0.65**	**1.13 ± 0.23**
3629	**12-MeC36**	0.32 ± 0.15	0.34 ± 0.21	0.24 ± 0.12	0.37 ± 0.21	**0.41 ± 0.17**	**0.22 ± 0.11**
3729	**11-;13-MeC37**	2.12 ± 0.9	2.21 ± 1.16	1.7 ± 0.6	2.42 ± 1.33	**2.62 ± 0.98**	**1.66 ± 0.43**
3751	**11,15-DiMeC37**	0.77 ± 0.32	0.79 ± 0.38	0.6 ± 0.18	0.89 ± 0.38	**0.96 ± 0.33**	**0.61 ± 0.25**
3774	5,15-;5,17-DiMeC37	0.91 ± 0.47	0.93 ± 0.53	0.7 ± 0.22	1.07 ± 0.56	1.11 ± 0.48	0.67 ± 0.24
3927	13-MeC39	0.81 ± 0.39	0.83 ± 0.51	0.62 ± 0.22	0.94 ± 0.62	0.98 ± 0.46	0.61 ± 0.25
3949	11,15-DiMeC39	0.62 ± 0.31	0.63 ± 0.35	0.49 ± 0.12	0.73 ± 0.39	0.73 ± 0.3	0.52 ± 0.1
3973	5,15-;5,17-DiMeC39	0.54 ± 0.28	0.56 ± 0.34	0.4 ± 0.13	0.65 ± 0.39	0.63 ± 0.31	0.43 ± 0.1

4. Discussion

Our aim was to increase our understanding of termite social immunity by carrying out a series of experiments that circumvented direct contact between nestmates and pathogens. By stimulating an internal immune response, our study reveals how collective responses towards pathogen-injected individuals varies according to the severity and timing of pathogen challenge, with first evidence that cannibalism in termites is triggered at an early stage during the internal host-pathogen interaction cycle; before pathogen viability or even terminal disease status is known.

By stimulating an internal immune response, we were also able to detect elevated rates of grooming at early time points following infection (as early as 2 hours p.i.). In comparison to previous work employing conidia on the termite cuticle (Chouvenc et al., 2009a; Davis et al., 2018; Esparza-Mora et al., 2020; Liu et al., 2019b; Myles, 2002; Rosengaus et al., 1998; Yanagawa et al., 2012; Yanagawa & Shimizu, 2007), our observed rates of grooming are lower across comparable time points, suggesting that direct contact with external pathogens may be more important in eliciting a robust allogrooming response. The benefits of allogrooming for enhancing group survival in the presence of external pathogens are well documented (Liu et al., 2019a; Rosengaus et al., 1998; Yanagawa et al., 2011; Yanagawa & Shimizu, 2007), which makes intuitive sense as allogrooming can remove potentially infectious microbes on the insect cuticle. When cuticular pathogens are absent, or once an internal infection has been initiated, allogrooming may serve a limited purpose. Overall, allogrooming may play an important role during the initial inspection phase of an individual, but its effectiveness as a collective response to an internally infected individual may be more restricted.

The general absence of burial supports the idea that termites preferentially eliminate potentially harmful individuals through cannibalism (Chouvenc & Su, 2012; Rosengaus & Traniello, 2001), although burial may be a more important defensive mechanism for older or decomposing cadavers (Chouvenc & Su, 2012; López-Riquelme & Fanjul-Moles, 2013; Sun et al., 2017). Indeed, as in previous studies, we observed a rapid transition to cannibalism correlating with visible signs of moribundity. Individuals that were very moribund at 15 hours following viable blastospore injection were cannibalized at a significantly higher rate than individuals injected with an equivalent dose of dead blastospores or a control solution. Our observations complement previous findings from fungus-exposed individuals that were cannibalized when *R. flavipes* termites were close to death (Davis et al., 2018).

Surprisingly, however, we also observed increases in cannibalism in response to both viable and dead blastospore challenge as early as 2 hours p.i., when termites did not yet display overt signs of illness. In fact, we found that cannibalism was generally triggered in response to individuals that were not necessarily terminally ill. This can be seen in focal termites injected with dead blastospores, which induced only 40% mortality at the individual level yet were nonetheless all eventually cannibalized by 24 hours p.i. when placed inside experimental colonies. This is despite the fact that dead blastospore injected termites did not display severe signs of sickness by this time. A similar pattern was observed when termites were injected with a dead blastospore solution lacking both fungal cells and intact destruxins (**Supplementary Figure S9, S10**), showing that even subcellular fungal components lacking active toxins are sufficient to elicit a cannibalism response.

This unexpected finding indicates that cannibalism is triggered prior to the onset of moribundity. This could reflect the need to react rapidly to the natural course of disease progression of entomopathogenic fungi such as *Metarhizium*, suggesting that *R. flavipes* might employ a “better safe than sorry” strategy towards individuals suspected of internal fungal

infection. It is possible that termites are responding to general behavioural and/or altered chemical cues (Konrad et al., 2012) associated with internal fungal pathogen presence, perhaps mediated by activation of innate immune signalling pathways (Liu et al., 2019a). *Metarhizium* deploys a multi-component infection strategy to kill the host and complete the infection cycle including toxin release after the fungus breaches the cuticular barrier (Gillespie et al., 2000), so once this stage has been reached, host death is likely unavoidable. There are several points during *Metarhizium* internal infection that could activate endogenous signalling pathways, which in turn may communicate a change in disease status to interacting nestmates. The first may be as soon as when blastospores have gained access to the hemocoel, as this could feasibly be linked to the rapid activation of innate immune signalling pathways. The second stage is associated with fungal acquisition of nutrients for growth and reproduction, during which time, termite hosts gradually begin to show visible signs of sickness (from 12 hours p.i.; **Supplementary Table S1**). Similar symptoms have been described from the higher termite *Nasutitermes exitiosus* (Hänel, 1982). Overlapping with this phase of fungal proliferation, is the ramping up of toxin biosynthesis (Gillespie et al., 2000; Iwanicki et al., 2020; Singh et al., 2016); (Chouvenc et al., 2009a; Roberts, 1966) which may be the proximal cause of host death (Hänel, 1982; Chouvenc et al., 2009a; Syazwan et al., 2021). Blastospores may evade innate insect immune responses via the reduction of the number of pathogen-associated molecular patterns (PAMPs) by camouflaging or modifying their β-glucans (for *M. anisopliae* collagen-like protein) (Tartar et al., 2005; Wang & St. Leger, 2006), but in general their interaction with the termite immune system remains unknown.

With respect to inactivated blastospores, these harbour several components that could activate the immune system of the host, including denatured cellular contents and cell wall constituents. In one study, heat-inactivated blastospores of the fungus *Beauveria bassiana* did not induce any observable priming effect (Vertyporokh et al., 2020) although injection of dead bacterial cells triggered an immune response in the same insect model (*Galleria mellonella*) (Wu et al., 2014). One possible factor to consider is that heat-inactivated *M. anisopliae* blastospores did not contain intact destruxins in our analysis. Such missing factors could represent a more important form of “danger” signal, either via direct interaction with signalling receptors, or indirectly via their negative influence on host cells, which may then produce signals of damage (Ming et al., 2014). We speculate that the faster cannibalism response to viable blastospore-injected termites may be linked to the presence and active synthesis of virulence factors such as destruxins, although this requires further testing.

We hypothesized that activation of endogenous pathways would result in external changes that may be perceptible by interacting nestmates. We examined cuticular hydrocarbon (CHC) profiles of immune-challenged individuals to understand whether they could serve as chemical cues indicating infection. Injections with both viable and inactivated blastospores showed a clear tendency to shift overall CHC profiles, with a trend towards more clear differentiation after a longer incubation time (**Figure 4**). More specifically, we detected significant increases in four exclusively methyl-branched CHCs 15 hours after injection with

viable blastospores compared to control-injected individuals (**Figure 5**). Interestingly, these four compounds are structurally related, long-chained mono- and di-methyl-branched alkanes with a similar chain length range (C35-C37) and internal methyl-branches at positions 11, 12, 13 and 15. A trend towards an increase in these compounds was also clearly discernible albeit non-significant in individuals injected with dead blastospores (**Figure 5**). These results might indicate a common pattern for encoding and conveying a chemical cue associated with infection status, which would be in line with the high potential of methyl-branched alkanes to encode chemical information (Chung & Carroll, 2015; Holman et al., 2010; Holze et al., 2021). Similar findings have been reported in challenged *Lasius* ants where infection has been linked with changes in CHCs of a similar chain length range (C33-35), albeit of non-methyl branched compounds (Pull et al., 2018) whereas in *Myrmica* ants, straight-chain alkane quantities increased upon infection while methyl-branched alkane quantities decreased (Csata et al., 2017). Studies reporting cuticular profile changes associated with disease have also been carried out in honeybees (Baracchi et al., 2012; Hernández López et al., 2017; Richard et al., 2008). In line with these findings, our study points to host condition-dependent cues as playing an important role, with elimination behaviours potentially being activated when changes in particular CHC compounds exceed a given quantitative threshold (Leclerc and Detrain, 2016). Our observations of increased amounts of long-chained mono- and di-methyl-branched alkanes over time following viable blastospore injection, as well as a trend towards higher quantities of these compounds in dead blastospore injected termites, are both consistent with a threshold hypothesis.

Overall, our results show that termites are capable of readily detecting and eliminating individuals displaying signs of an internal fungal challenge. Our findings also indicate that elimination response behaviours are triggered before individuals enter a terminal disease state, and that individuals that could theoretically recover, will nonetheless be cannibalized by their nestmates. This finding may reflect the need for a vigilant social immune system that has evolved to recognize early signs of a typically highly virulent fungal infection. In terms of the care-kill paradigm, internal immune challenge with *M. anisopliae* appears to be uniformly associated with a kill response. Trade-offs in immune system vigilance have been explored in the context of beneficial versus pathogenic microbiota (Metcalf & Koskella, 2019) and similar trade-offs may also operate with regards to social immunity. Here, the level of social immune vigilance might be determined by pathogen hazard on the one hand, and cost of social immunity ("damage" to the colony due to elimination of constituent members as well as risk to the survival of intervening nestmates), on the other. As in individual immunity, a positive correlation between pathogen hazard and social immune vigilance may be expected. It is perhaps unsurprising that *R. flavipes* does not differentiate between viable and inactivated blastospores or associated moribundity effects arising from an internal *M. anisopliae* challenge, because such a situation would not arise under natural conditions. These findings suggest that the cues employed to trigger elimination are generated during an early phase of internal challenge, with potential for early crosstalk between immune receptor activation and other physiological processes such as metabolism and the regulation of biosynthetic pathways

involved in CHC variation. Expansion of our chemical analysis to other volatile compound classes such as alcohols (e.g. Butanol, 2,3-Butanediol) and ketones (e.g. 2-Pentanone) released by entomopathogenic fungi (Bojke et al., 2018; Hummadi et al., 2021) may also be warranted. Another interesting question to consider is whether modification of the cuticular profile in immune-challenged individuals is sufficient to induce antifungal immunity in interacting nestmates (Baracchi et al., 2012; Hernández López et al., 2017; Nielsen & Holman, 2012; Pull et al., 2018; Richard et al., 2008). Understanding the molecular basis of these processes represents a promising avenue for further study. Other targets for future work could include functional experiments to test whether specific compounds, chemical combinations, or general alterations in overall CHC abundance are sufficient to trigger changes in social immune behaviour.

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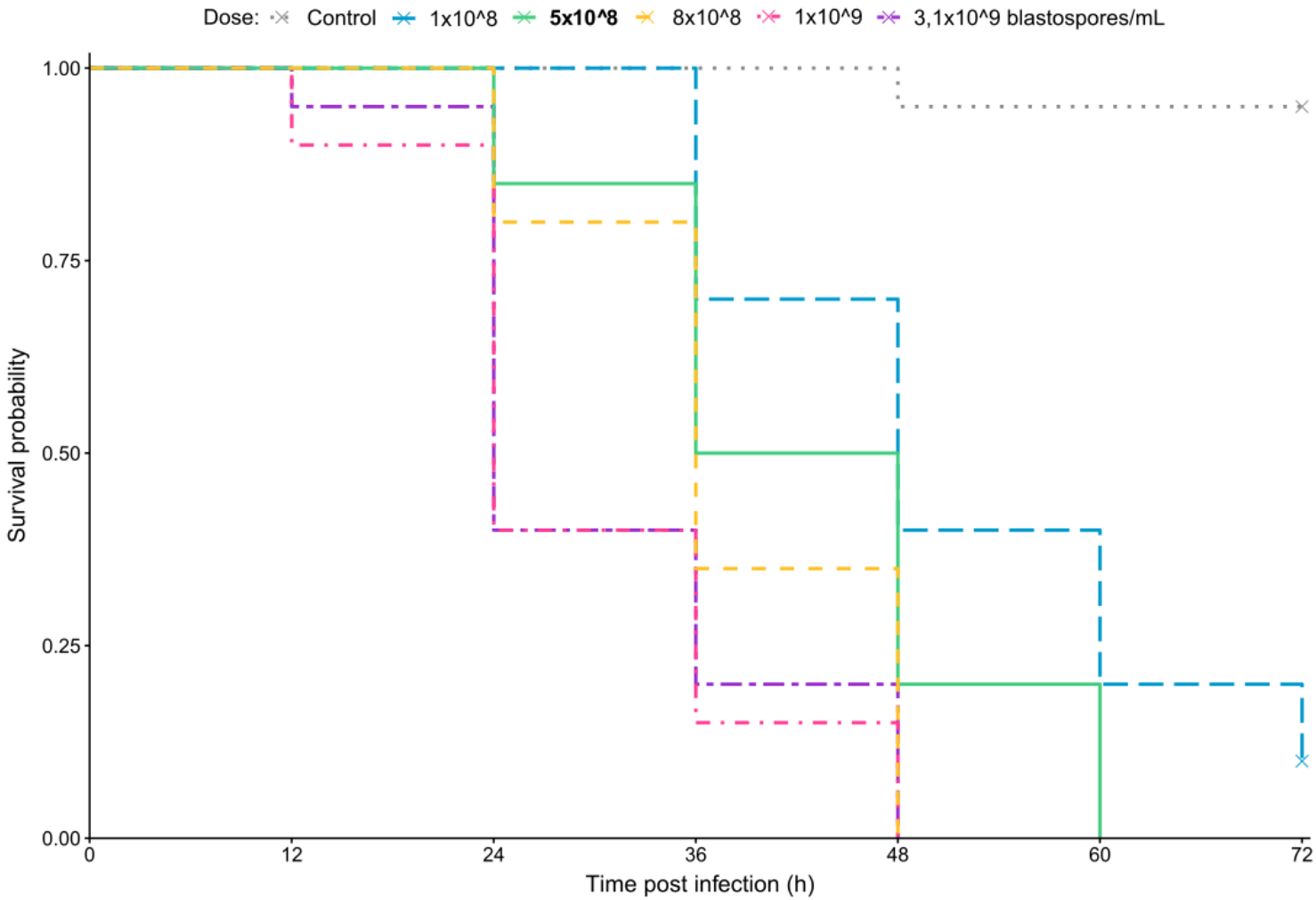
Supplementary Material

Survival assay of different doses of *M. anisopliae* blastospores

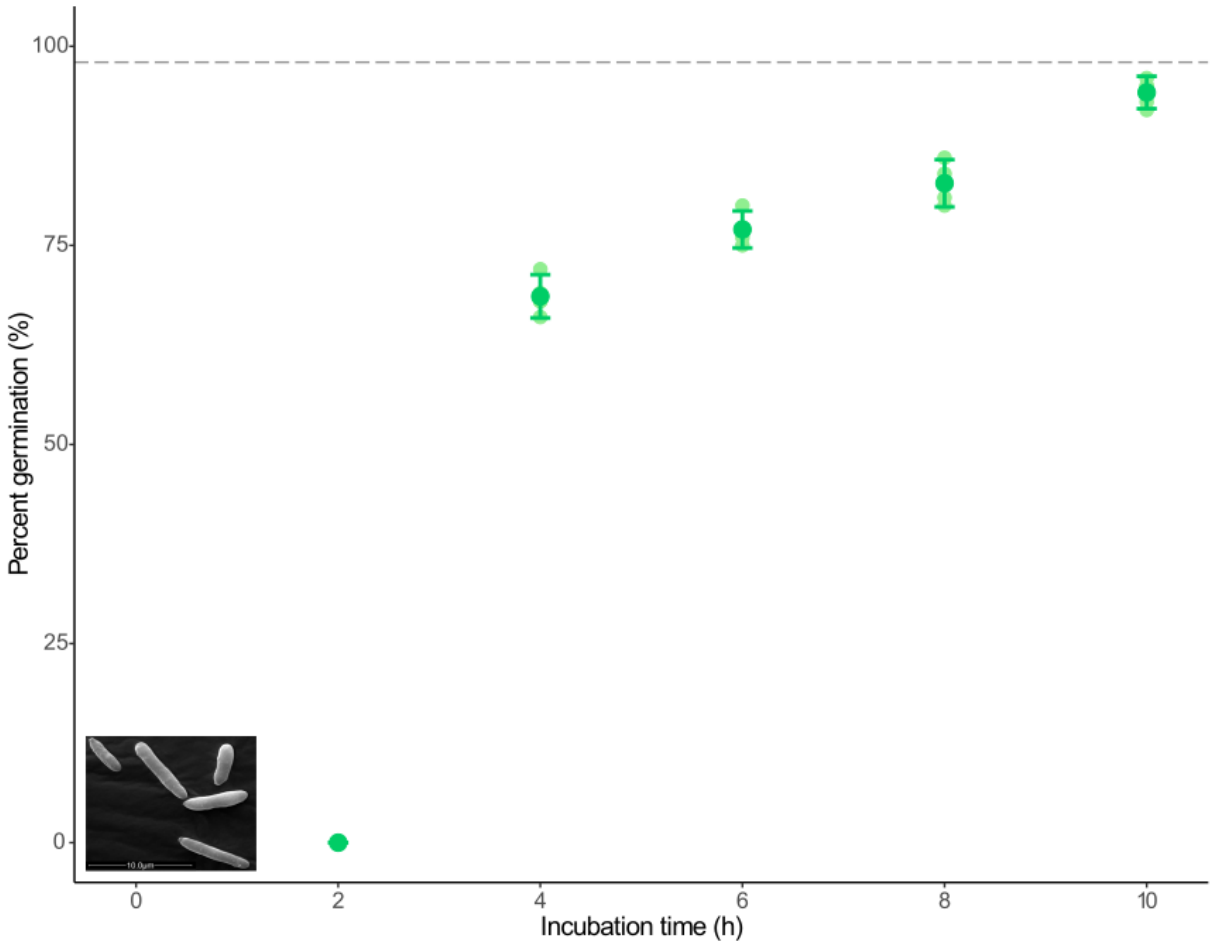
To select pathogen dose, 120 *Reticulitermes flavipes* workers from colony 5 (20 termites/treatment) were anaesthetized with CO_2, then injected with 41,4 nL of five different concentrations of viable blastospores (1×10^8, 5×10^8, 8×10^8, 1×10^9 and $3{,}1 \times 10^9$ blastospores/mL) of *Metarhizium anisopliae* and Ringer solution (Control) directly into the hemocoel using a Nanoject II (Drummond Scientific Company, USA). Infected termites were kept individually in small Petri dishes (35 mm), each containing a Pall cellulose pad moistened with 1 mL of distilled water and then incubated in darkness for a total of 72 hours at 27 °C. Infected termites were inspected every 12 hours to determine survival.



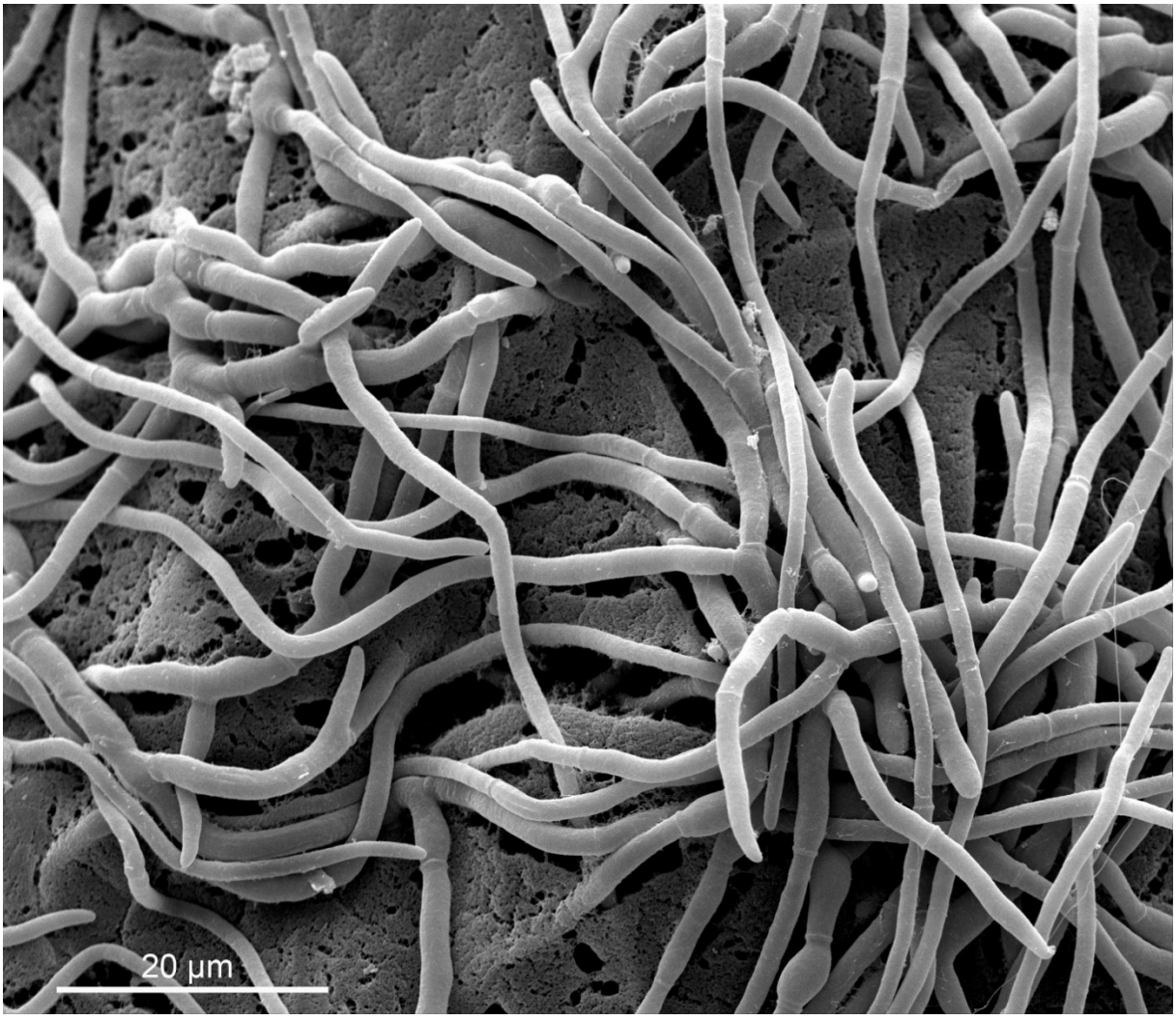
Supplementary Figure S1. Survival analysis of the different doses of viable blastospores per mL represented by different colors. The x axis indicates time post injection (in hours) while the y axis depicts survival probability, calculated as the proportion of termites from every dose group that were not dead. Crosses indicate the presence of right-censored data (i.e. termites that were not dead during the experiment). In bold (5×10^8 blastospores/mL) represent the selected dose for our experiments.

Germination experiment

To evaluate germination in vitro, 5 PDA plates were streaked with the 5×10^8 blastospores/mL *Metarhizium anisopliae* suspension, parafilmed, and incubated in the dark at 25 °C. At 0, 2, 4, 6, 10 and 12 hours after inoculation, at least 100 blastospores per plate were evaluated for germination at 200-400× magnification using a phase contrast microscope. After 12 hours, dense hyphal growth made it impossible to calculate the germination rate. Blastospores were considered germinated when an elongating germ tube of any size was visible. Means and 95% confidence intervals were calculated and plotted using R (version 4.0.2).



Supplementary Figure S2. Germination of blastospores on PDA plates. Larger dots represent means, smaller dots are individual data points, and bars show 95% confidence intervals.



Supplementary Figure S3. SEM image of *M. anisopliae* blastospore germination at 15 hours post-inoculation.

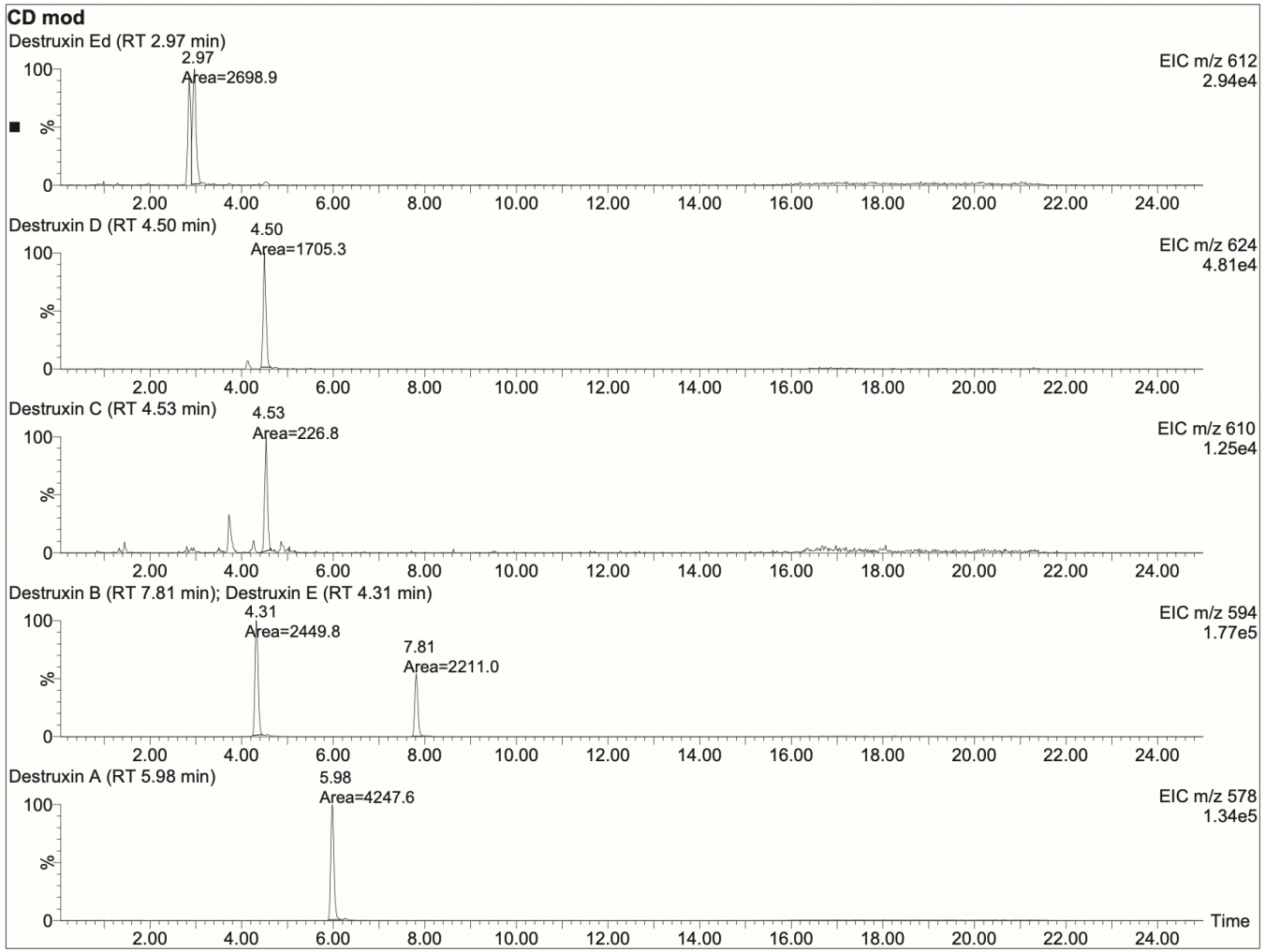
Sickness cue experiment

To determine the incubation time points, 5 *Reticulitermes flavipes* workers from each colony (11+13, X and 5) were anaesthetized with CO_2, then injected with 41.4 nL of a suspension of either viable or dead blastospores (5×10^8 blastospores/mL) of *Metarhizium anisopliae* and Ringer solution directly into the hemocoel using the Nanoject II. Injection with dead blastospores was carried out in colony 11+13. Infected termites were kept individually in small Petri dishes (35 mm), each containing a Pall cellulose pad moistened with 1 mL of distilled water and then incubated in darkness for a total of 48 hours at 27 °C. Infected termites were checked carefully every 2 hours to determine health status.

Supplementary Table S1. Description of the symptoms caused by *M. anisopliae* when blastospores are injected directly into the termite hemocoel. Different colors (light blue, pink, green and yellow) represent varying signs of disease observed after injection of blastospores. "Signs of weakness"; "Very sick/moribund"; "close to death" refer to the following behaviours, respectively: slower walking; hardly able to walk; antennal movements only or legs if lying dorsally.

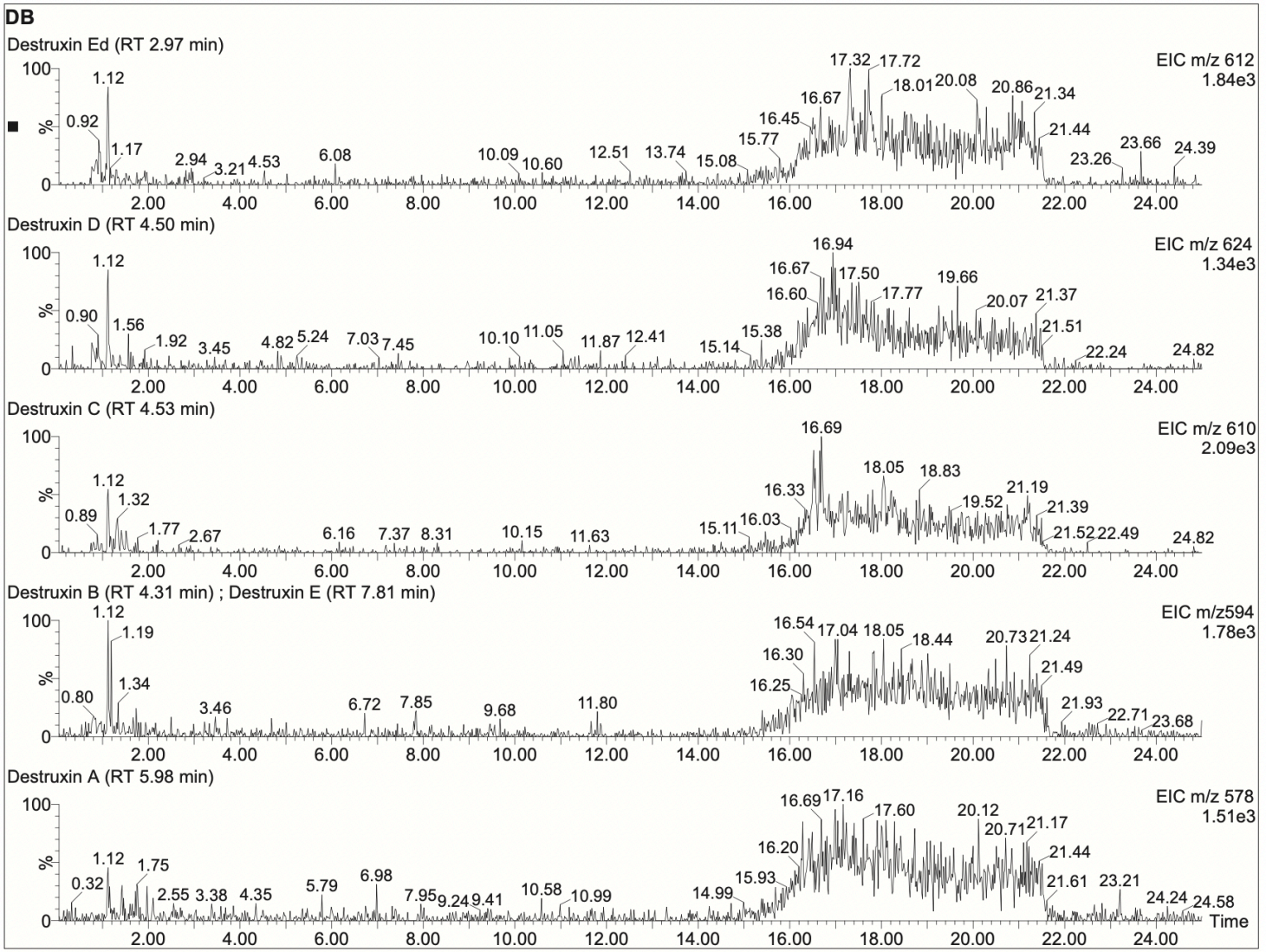
Incubation time	**Description: Viable blastospores**				**Dead blastospores**	
	Control	Colony 11+13	Colony X	Colony 5	Control	Colony 11+13
0 h	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection
2 h	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection
4 h	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection
6 h	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection
8 h	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection
10 h	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection	No signs of infection
12 h	No signs of infection	First signs of weakness	First signs of weakness	First signs of weakness	No signs of infection	No signs of infection
14 h	No signs of infection	~	~	~	No signs of infection	No signs of infection
16 h	No signs of infection	Sick and sluggish	Sick and sluggish	Sick and sluggish	No signs of infection	No signs of infection
18 h	No signs of infection	Very sick / moribund	Sick and sluggish	Sick and sluggish	No signs of infection	No signs of infection
20 h	No signs of infection	Close to death	Sick and sluggish	Very sick / moribund	No signs of infection	No signs of infection
22 h	No signs of infection	Termites start dying	Very sick / moribund	Close to death	No signs of infection	First signs of weakness
24 h	No signs of infection		Close to death	Termites start dying	No signs of infection	Look weak
26 h	No signs of infection		Termites start dying		No signs of infection	Sick and sluggish
36 h	No signs of infection				No signs of infection	Termites start dying

Destruxin Analysis

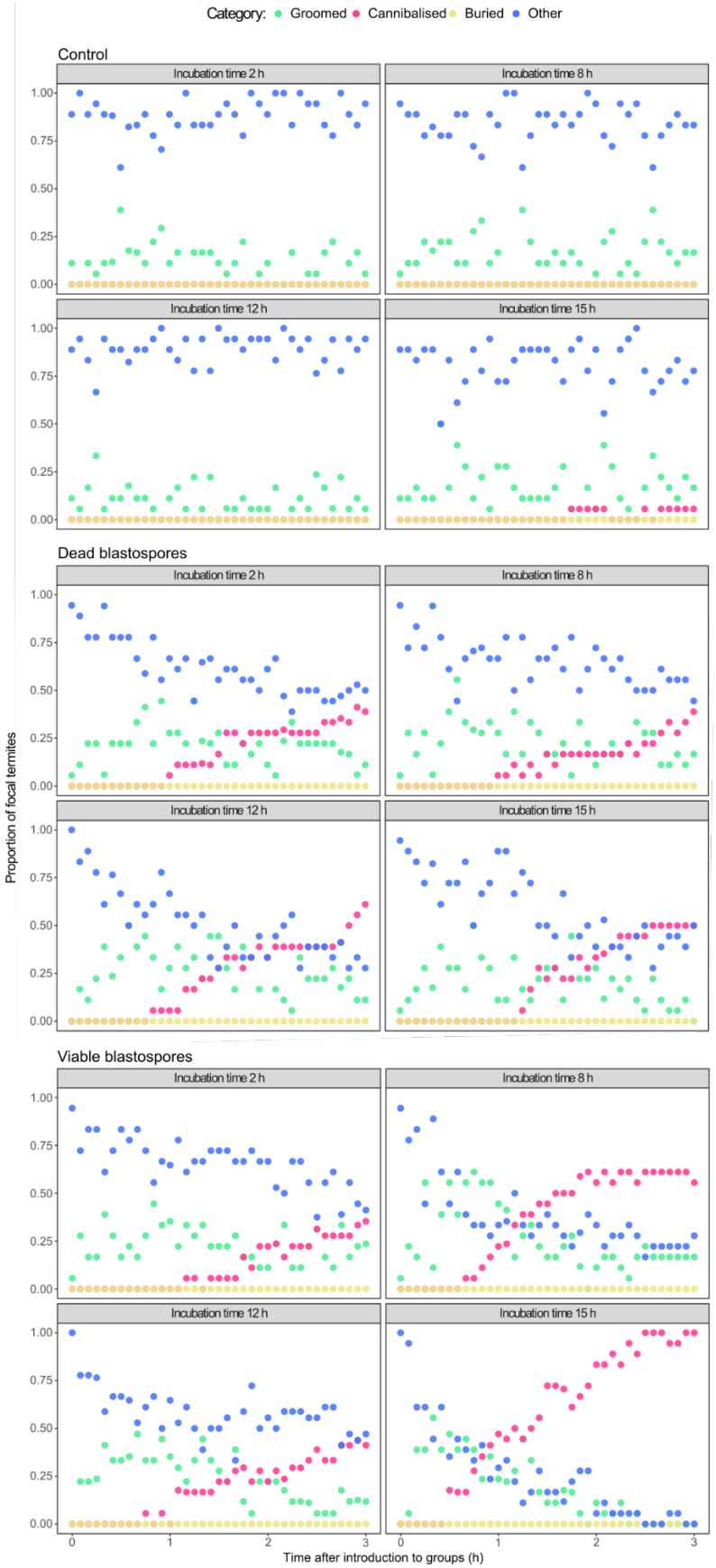


Supplementary Figure S4. Chromatograms of 5 classes of destruxins in the blastospore culture media: destruxin Ed (m/z= 612), destruxin D (m/z= 624), destruxin C (m/z= 610), destruxin B (m/z= 594) and destruxin A (m/z= 578). m/z ratios for searching for the respective destruxins were obtained from Jegorov et al. (1998)

and Taibon et al. (2014). Additionally, structural assignment of destruxins via comparison of MS/MS spectra with reported fragment ions was performed with LC and MS conditions similar to the ones reported in Taibon et al. (2014).



Supplementary Figure S5. Chromatograms of the filtrate of autoclaved blastospores. None of the 5 classes of destruxins were detectable.



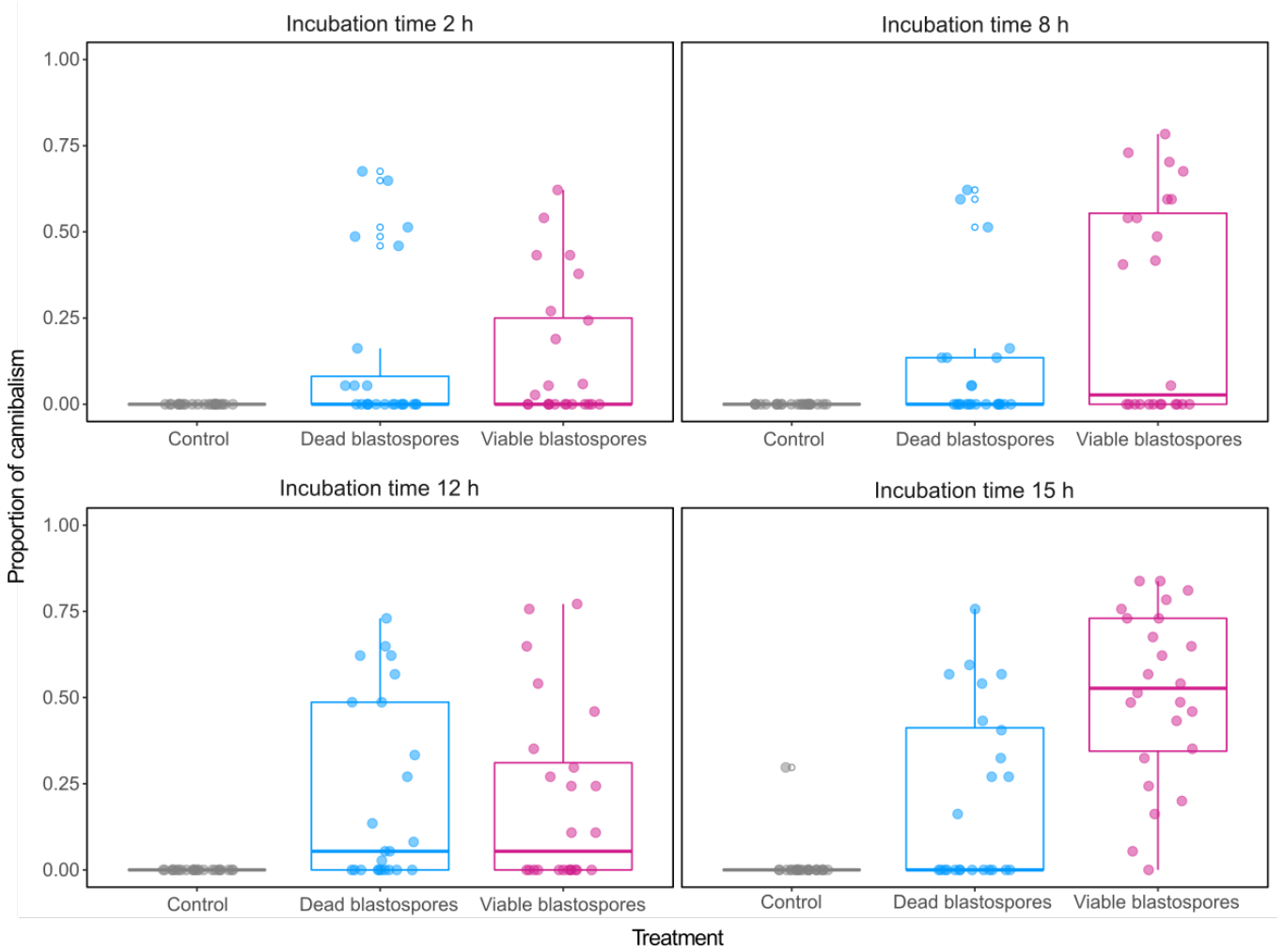
Supplementary Figure S6. The transition of the different behaviours over time during the 3-hours observation period for each of the 3 treatments: control, dead blastospores and viable blastospores. Each point represents the proportion of focal termites that were observed in a given category during a scan.

Supplementary Table S2. z and p values from post hoc pairwise comparisons (Tukey tests using a Bonferroni correction) of the proportion of grooming over all observed behaviours. Statistically significant differences are indicated in bold. Empty squares mean that the comparison has already been made and can be found somewhere else in the table.

Incubation time 2 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 3.293** ***P* = 0.00297**		
Viable blastospores (Blastospores+)	**z = 4.448** ***P* = 2.6e-05**	z = 1.247 *P* = 0.63739	
Incubation time 8 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	z = 2.088 *P* = 0.110		
Viable blastospores (Blastospores+)	z = 2.117 *P* = 0.103	z = 0.257 *P* = 1.000	
Incubation time 12 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 4.324** ***P* = 4.61e-05**		
Viable blastospores (Blastospores+)	**z = 4.515** ***P* = 1.90e-05**	z = 0.170 *P* = 1	
Incubation time 15 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	z = 1.480 *P* = 0.417		
Viable blastospores (Blastospores+)	z = 1.359 *P* = 0.522	z = 0.224 *P* = 1.000	

Supplementary Table S3. z and p values from post hoc pairwise comparisons (Tukey tests using a Bonferroni correction) of the probability of not being cannibalised. Statistically significant differences are indicated in bold. Empty squares mean that the comparison has already been made and can be found somewhere else in the table.

Incubation time 2 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 3.114** ***P* = 0.00553**		
Viable blastospores (Blastospores+)	**z = 3.312** ***P* = 0.00277**	z = 0.328 *P* = 1.00000	
Incubation time 8 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 2.397** ***P* = 0.04965**		
Viable blastospores (Blastospores+)	**z = 3.226** ***P* = 0.00377**	z = 1.725 *P* = 0.18504	
Incubation time 12 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 2.946** ***P* = 0.00967**		
Viable blastospores (Blastospores+)	**z = 2.808** ***P* = 0.01495**	z = -0.299 *P* = 1.00000	
Incubation time 15 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Viable blastospores (Blastospores+)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 2.684** ***P* = 0.021822**		
Viable blastospores (Blastospores+)	**z = 4.822** ***P* = 4.26e-06**	**z = 4.054** ***P* = 0.000151**	

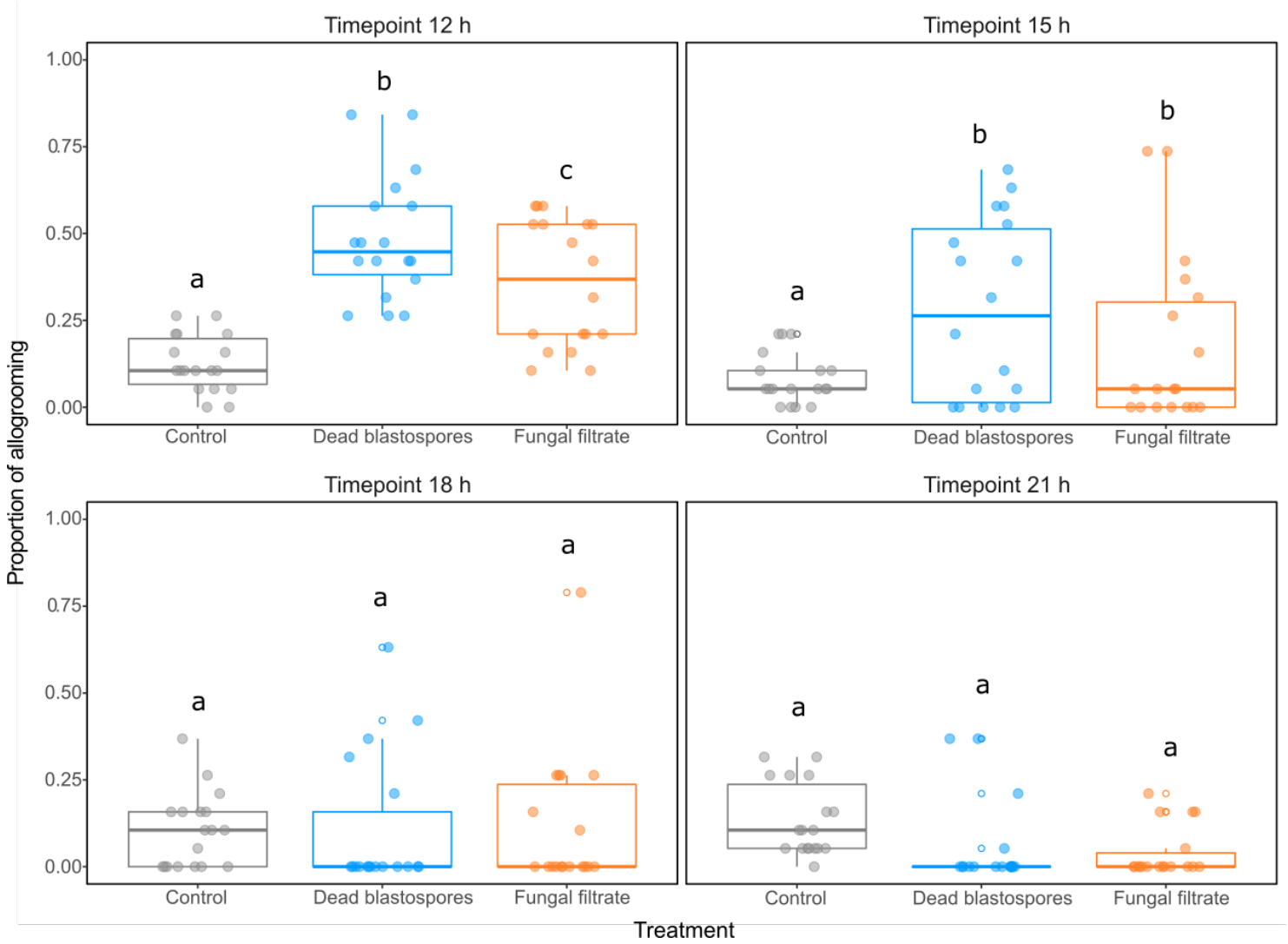


Supplementary Figure S7. Cannibalism as a proportion of total visible states across treatments. Lower and upper hinges correspond to first and third quartiles, the upper whisker extends to the largest value if it is no greater than 1.5 times the inter-quartile rage from the hinge, and the lower whisker extends to the smallest value if it is no smaller than 1.5 times the inter-quartile range from the hinge.

Behavioural Experiment with Fungal Filtrate

Supplementary Table S4. z and p values from post hoc pairwise comparisons (Tukey tests using a Bonferroni correction) of the proportion of grooming over all observed behaviours. Statistically significant differences are indicated in bold. Empty squares mean that the comparison has already been made and can be found somewhere else in the table.

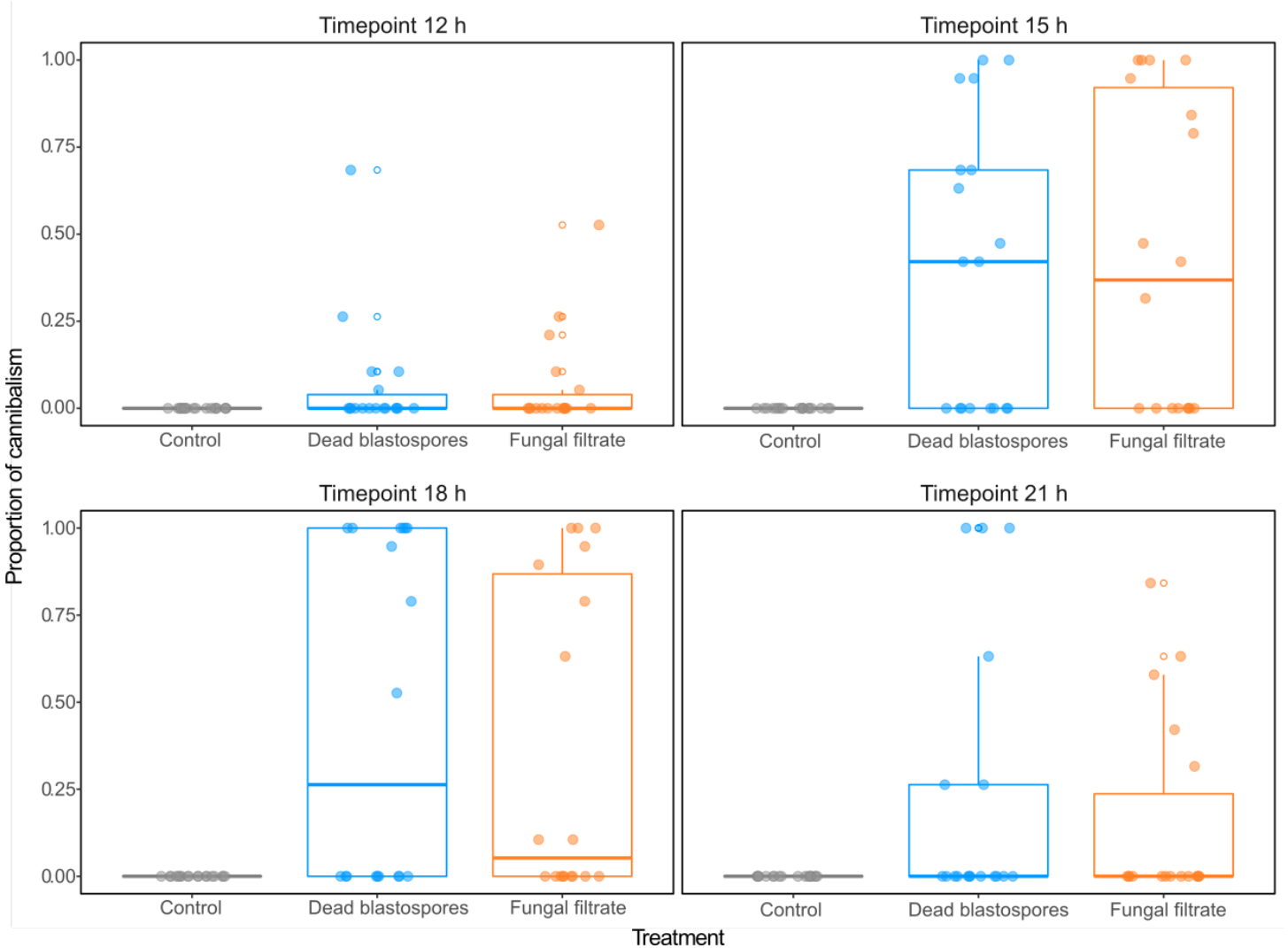
Time point 12 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Fungal filtrate (Filtrate)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 7.636** ***P* = 6.73e-14**		
Fungal filtrate (Filtrate)	**z = 5.349** ***P* = 2.66e-07**	**z = -2.510** ***P* = 0.0363**	
Time point 15 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Fungal filtrate (Filtrate)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 6.626** ***P* = 1.04e-10**		
Fungal filtrate (Filtrate)	**z = 4.634** ***P* = 1.08e-05**	z = -2.003 *P* = 0.135	
Time point 18 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Fungal filtrate (Filtrate)
Control (Ringer)			
Dead blastospores (Blastospores-)	z = 0.670 *P* = 1.000		
Fungal filtrate (Filtrate)	z = 1.628 *P* = 0.311	z = 0.765 *P* = 1.000	
Time point 21 h			
Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Fungal filtrate (Filtrate)
Control (Ringer)			
Dead blastospores (Blastospores-)	z = -1.967 *P* = 0.1476		
Fungal filtrate (Filtrate)	z = -2.206 *P* = 0.0822	z = -0.093 *P* = 1.0000	



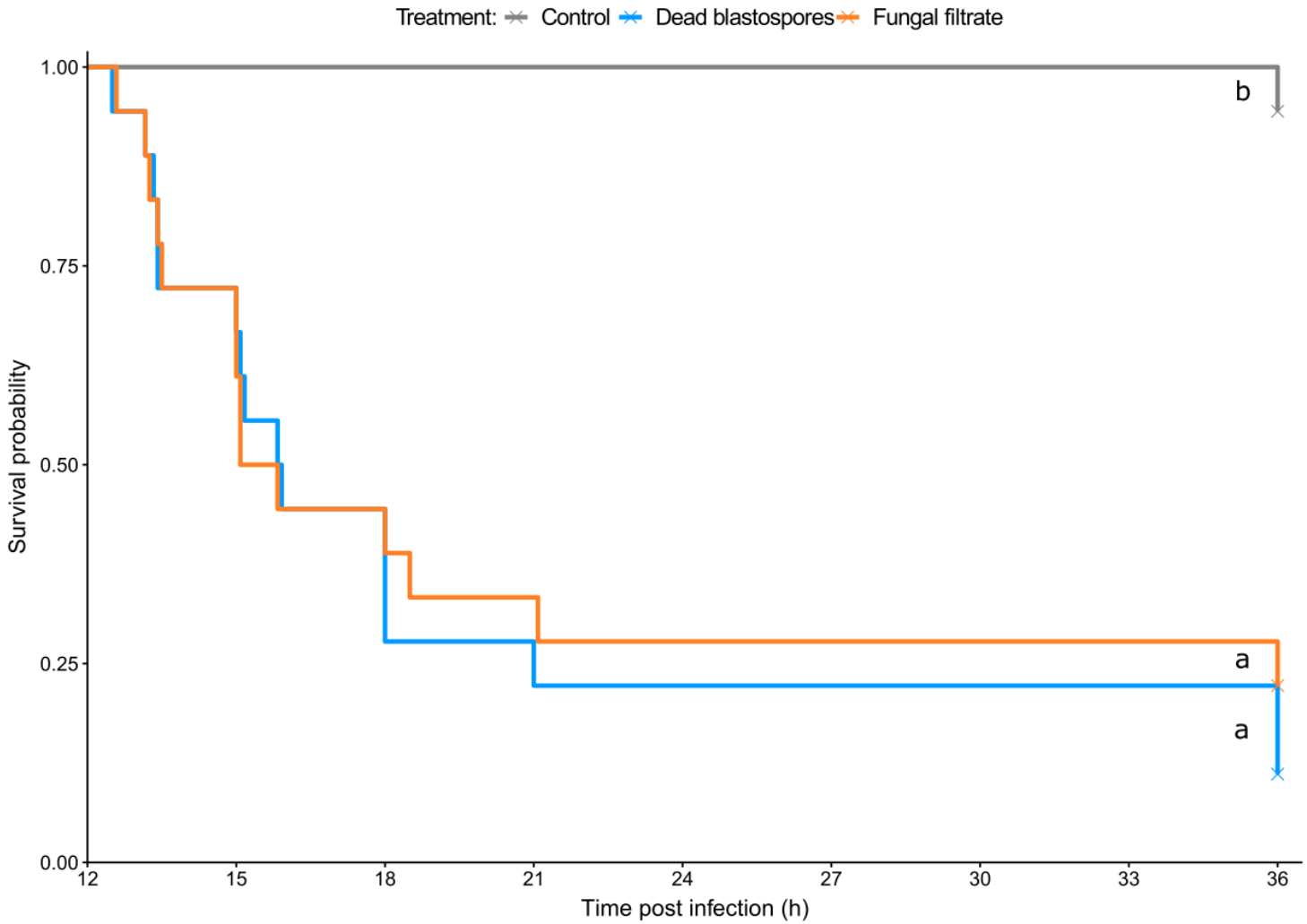
Supplementary Figure S8. Allogrooming as a proportion of total states over time for every treatment (marked with different colors). Treatments marked with different letters were significantly different. Lower and upper hinges indicate first and third quartiles, respectively. The bold middle line represents the median. Whiskers extend to the smallest/largest value if not smaller/greater than 1.5 times the interquartile range (box length).

Supplementary Table S5. z and p values from post hoc pairwise comparisons (Tukey tests using a Bonferroni correction) of the survival curves indicating the probability of being cannibalized. Statistically significant differences are indicated in bold. Empty squares mean that the comparison has already been made and can be found somewhere else in the table.

Treatment	Control (Ringer)	Dead blastospores (Blastospores-)	Fungal filtrate (Filtrate)
Control (Ringer)			
Dead blastospores (Blastospores-)	**z = 3.587** ***P* = 0.00100**		
Fungal filtrate (Filtrate)	**z = 3.339** ***P* = 0.00253**	z = -0.732 *P* = 1.00000	



Supplementary Figure S9. Cannibalism as a proportion of total visible states across treatments. Lower and upper hinges correspond to first and third quartiles, the upper whisker extends to the largest value if it is no greater than 1.5 times the inter-quartile rage from the hinge, and the lower whisker extends to the smallest value if it is no smaller than 1.5 times the inter-quartile range from the hinge.



Supplementary Figure S10. Survival analysis of the different treatment's groups represented by different colors. The x axis indicates time post injection (in hours) while the y axis depicts survival probability, calculated as the proportion of focal termites from every treatment group that were not cannibalised. Crosses indicate the presence of right-censored data (i.e. focal termites that were not cannibalised during the experiment). A clear difference in survival is visible between the control and the fungal treatments (dead blastospores, fungal filtrate). Treatments marked with the same letter were not significantly different.

Chapter II

A comparison of social immune responses and cuticular chemistry changes induced by fungal and bacterial challenge in a termite

A comparison of social immune responses and cuticular chemistry changes induced by fungal and bacterial challenge in a termite

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Author Contributions

DPM and MAEM conceived the overall idea. MAEM, TL, SJ, VP and RB designed the experiments and collected the data. JB performed chemical analysis of termite CHCs and collected the data. MAEM, JB and DPM wrote the manuscript. All authors contributed critically to the drafts.

Unpublished manuscript.

Abstract

To explore how social insects manage diverse infectious diseases, including when an infection poses a serious threat to the colony, we carried out a comparative analysis of the interactions between *R. flavipes* and two different pathogens: the entomopathogenic bacteria *Pseudomonas entomophila* and the entomopathogenic fungus *Metarhizium anisopliae*. Termite workers were injected with different doses of either viable or heat-killed bacteria, viable or heat-killed fungus or Ringer´s solution to examine similarities and differences in the social immune responses to different microbial pathogens and characterize associated cuticular hydrocarbon (CHC) changes. We found evidence that collective defences in *R. flavipes* vary according to infection type and severity. Allogrooming behaviour was expressed initially and were progressively replaced by cannibalism across all lethal doses, regardless of severity and pathogen type. Interestingly, the rate of transition to cannibalism was significantly slower in the equivalent bacterial challenge of intermediate severity, indicating that social immune responses are less sensitive to bacterial versus fungal challenge. Distinct infection modes linked to bacterial and fungal pathogens or contrasting evolutionary exposures to this termite species may underlie these differences but disentangling the precise cause of this difference requires further functional comparative studies. Further to this, we found that regardless of pathogen type, moribundity appears unnecessary as a trigger for termite cannibalism, suggesting that a threshold of immune or other physiological activation may instead be key to eliciting specific social immune behaviours. Analysis of CHC profiles from termites injected with different fungal and bacterial doses revealed unique patterns of CHCs associated with different pathogens and challenge intensities, with a discriminant analysis showing a particularly distinct profile in termites injected with viable blastospores; which was the treatment associated with the strongest cannibalism response.

1. Introduction

Insects have effective immune systems that can protect hosts against infection from a wide array of pathogens. Yet termites, which live in crowded colonies of closely related individuals within microbially-rich environments (Rosengaus et al., 1998, 1999b, 2011; Chouvenc & Su, 2010, 2012; Chouvenc et al., 2011) appear to mount a relatively weak immune response to bacterial and fungal pathogen challenge at the individual level (He et al., 2021; Avulova and Rosengaus, 2011), yet collectively they are highly resilient to disease outbreak at the colony level (Chouvenc & Su, 2012; Liu et al., 2019b). In recent years, termites have emerged as a powerful model for the study of host-pathogen interactions and insect immunity (Chouvenc & Su, 2010). An attractive feature of the termite study system is the existence of multiple disease defence mechanisms that cut across both individual and social immunity (Cremer et al., 2007, 2018; Liu et al., 2019b; Bulmer et al., 2010, 2019) and which protect against the transmission of pathogens within colonies (Rosengaus et al., 2011; Davis et al., 2018; Bulmer et al., 2019; Chouvenc & Su, 2012). At the individual level, termites can defend against pathogens through innate physiological and biochemical defences, such as the cellular and humoral response (Hamilton & Bulmer, 2012; Chouvenc et al., 2009; He et al., 2021). At the colony level, termites complement individual immunity with effective collective defence processes otherwise termed social immunity. Nestmates can engage in vibratory alarm behaviour (Rosengaus et al., 1999a; Myles, 2002; Bulmer et al., 2019), carry out allogrooming (Rosengaus et al., 1998; Yanagawa & Shimizu, 2007; Davis et al., 2018; Liu et al., 2019a), produce antimicrobial secretions to disinfect body and nest surfaces (Hamilton et al., 2011; He et al., 2018; Bulmer et al., 2019) engage in destructive disinfection (elimination) behaviours (Rosengaus & Traniello, 2001; Davis et al., 2018; Esparza-Mora et al., 2020; Chouvenc & Su 2012) and bury infected cadavers (Kramm et al., 1982; Chouvenc & Su 2012; Chouvenc et al., 2012; López-Riquelme & Fanjul-Moles, 2013). Given the wide repertoire of adaptive defence mechanisms that termites can deploy against infectious threats, it is likely that diseases have acted as major selective pressures during the evolution of termites (Traniello et al., 2002).

Allogrooming is a particularly important early defensive mechanism because it helps to remove pathogens from cuticles and enables the application of antimicrobial disinfectants (Yanagawa & Shimizu 2007; Hamilton & Bulmer, 2012; Davis et al., 2018; Liu et al., 2015, 2019a). However, when allogrooming fails or when an infection has advanced to the point where the pathogen has successfully penetrated the cuticle and invaded the body cavity of the host (Hamilton & Bulmer, 2012; Chouvenc et al., 2009; Avulova & Rosengaus, 2011), allogrooming can only play a limited role, whereas cannibalism can effectively contain an infectious threat through the elimination of the diseased individual (Rosengaus & Traniello, 2001; Chouvenc et al., 2008; Chouvenc & Su 2012; Davis et al., 2018; Esparza-Mora et al., 2020). This care-kill dichotomy has been well described in diverse insect societies (Cremer et al., 2018; Cremer & Sixt, 2009), yet understanding of its mechanistic underpinnings remain largely lacking. It has been suggested that the onset of cannibalism coincides with the appearance of post-infection signs of moribundity, which may be important signals of the

insect host's eventual death (Yanagawa et al., 2011; Davis et al., 2018). Experiments simulating internal fungal infections reveal significant chemical alterations to the cuticle of challenged individuals, indicating that the host itself may play a role in signalling its own demise (Chapter I). A related issue is whether social insects use cues derived primarily from the host or from the pathogen, or a combination of both, when responding to infected individuals. Finally, the nature of the triggering process is unclear. One hypothesis posits that responses are initiated by the crossing of a certain threshold of a given cue or set of cues (Masterman et al., 2001; Gramacho et al., 2003; Grüter et al., 2018).

We explore these questions by investigating termite social immune responses to two microbial pathogens and correlating these with chemical changes on the cuticle of immune-challenged individuals. Specifically, we ask whether a novel bacterial pathogen can elicit similar chemical changes on the surface of challenged hosts as an established fungal pathogen. We then explore the extent to which chemical changes are correlated with collective behavioural responses to the novel and the adapted pathogen, respectively. Several studies have explored collective termite defence strategies against fungal pathogens, but less is known about termite responses to bacterial challenges. In the present study, we used the subterranean termite *Reticulitermes flavipes* and two different entomopathogenic microorganisms: the bacterial pathogen, *Pseudomonas entomophila*, a Gram-negative non-native facultative pathogen, originally described from *Drosophila* (Liehl et al., 2006) which is able to infect a variety of other insects (Vallet-Gely et al., 2010; Sieksmeyer et al., 2019, in review), and the fungus *Metarhizium anisopliae*, a natural termite pathogen that has been widely employed in the study of termite social immunity (Hänel, 1982; Rosengaus et al., 1998; Chouvenc & Su, 2010; Liu et al., 2019b; Davis et al., 2018; Zoberi, 1995; Syazwan et al., 2021). The *P. entomophila* genome reveals the existence of a large set of genes encoding putative virulence factors (Liehl et al., 2006), which make it a useful bacterial pathogen to compare with *M. anisopliae*, which is also a facultative pathogen with a rapid toxin-mediated infection strategy (Vega et al., 2012).

Our knowledge of termite social immunity is predominantly based on studies using external infections of fungal pathogens that penetrate the insect cuticle. A limited number of studies have explored interactions between termites and internal bacterial infections, but these have focused on immune responses occurring at the individual level (Rosengaus et al., 1999b; Hussain et al., 2013; Luo et al., 2022). Our study takes a different approach by seeking to compare the collective behavioural responses towards nestmates infected with a native versus a non-native entomopathogen and correlating these with cuticular chemical changes of challenged individuals. We test the following hypotheses: i) collective responses, including elimination behaviours (cannibalism) are greater towards native (*M. anisopliae*) versus non-native (*P. entomophila*) challenge at equivalent severities of infection; ii) corresponding chemical changes are more extensive in individuals challenged with the native versus the non-native pathogen at equivalent infection severities. By comparing across pathogens, we aim to

enhance knowledge of the social immune mechanisms that underpin the collective defensive decision making of termites.

2. Materials and methods

2.1. Termites

Three colonies of the subterranean termite *R. flavipes* were used in these experiments: colonies 11+13, 5, and X. Pieces of wood containing dense aggregations of termites belonging to these colonies were collected from the field. Colonies 5 and 11+13 were collected in Île d'Oléron, France, in 1994 and 1999 respectively and maintained in a dark room at 26 °C, 84% humidity. Colony X was collected in Soulac-sur-Mer, France, in 2015 and maintained in a dark room at 28 °C, 83% humidity. As described by Becker (Becker, 1969), all colonies were kept in separate sheet metal tanks located at the Federal Institute for Materials Research and Testing (Bundesanstalt für Materialforschung und –prüfung) in Berlin, Germany, and had access to wood as well as sufficient damp soil to burrow. Cardboard baits were used to extract termites from their parental colonies according to Tracy (2003). Upon extraction, termites from the same colony were transferred to plastic boxes containing cellulose pads (Pall Corporation, Port Washington, USA) that had been moistened with tap water. Collected termites were kept at the same temperature as the parent colony until staining or transfer to experimental Petri dish nests.

2.2. Fungal pathogen

We used the obligately killing pathogen of *R. flavipes* termites, *Metarhizium anisopliae* (DSM 1490) (Zoberi, 1995; Chouvenc and Su, 2012; Hänel, 1982; Syazwan et al., 2021). Prior to each experiment, the conidia were grown on potato dextrose agar (PDA) at 25 °C in the dark until sporulation. For the preparation of conidia suspension, we followed the protocol established by Davis et al. with no alterations (Davis et al., 2018). The germination rate of conidia and blastospores was determined as described for (Chapter I) and was >90% in all cases. Blastospores are a single-cell spore stage produced within the infected host to facilitate the spread of the infection and evade insect immune responses (Syazwan et al., 2021). Blastospores were cultured by adding 1 mL of conidia suspension (1 x 10^8 conidia/mL) to 100 mL of Adámek´s and modified liquid media in a 300 mL Erlenmeyer flask, which was then incubated (3 days, 290 rpm, 25 °C) (Adámek, 1965; Chapter I). After incubation, the blastospores were harvested by filtering the culture media through two layers of a sterile miracloth (Merck KGaA, Darmstadt, D). The resulting blastospore suspension was then washed (5 min, 2000 g, 4 °C) three times in Ringer´s ¼ solution (Chapter I). Just as for conidia, blastospores concentration was determined in a Thoma counting chamber and adjusted to 5 x 10^6 and 5 x 10^8 blastospores/mL. Since our aim was to understand how generalized social immune responses act against different infection types, blastospores were either alive (viable blastospores; 5 x 10^8 blastospores/mL: 100% mortality) or killed by heat (dead blastospores;

0% germination and 5 x 10^8 blastospores/mL: 40% mortality, 5 x 10^6 blastospores/mL: 0% mortality). Control treatment consisted of sterile Ringer´s ¼ solution only. Dead blastospore suspension preparation and germination assessment were carried out as described elsewehere (Chapter I).

2.3. Bacterial pathogen

We used the Gram-negative bacterial pathogen *Pseudomonas entomophila* (strain L48; DSM 28517), which is not a known natural pathogen of blattodean insects but is nonetheless capable of infecting them (Sieksmeyer et al., 2019, in review). Bacteria were stored at -70 °C until use in experiments. One bead of frozen *P. entomophila* was inoculated in 20 mL of nutrient broth. The culture was incubated overnight at 140 rpm and 28 °C. 150 µL of the overnight culture of *P. entomophila* was mixed in 10 mL fresh nutrient medium and incubated at 28 °C and 140 rpm. Bacteria were grown to exponential phase standardized by optical density of 0.68 at 600 nm, representing 1.8 x 10^8 CFUs (determined by plating) (Sieksmeyer et al., 2019, in review). Bacterial suspensions were obtained by diluting bacteria in Ringer´s ¼ solution (5 x 10^5, 3 x 10^4 and 1 x 10^8 CFUs/mL). An aliquot of the 1 x 10^8 CFUs/mL suspension was autoclaved to inactivate the bacterial cells. Since our aim was to understand how generalized social immune responses act against bacterial infections, bacteria were either alive (viable bacteria; 5 x 10^5 CFUs/mL: 100% mortality and 3 x 10^4 CFUs/mL: 40% mortality) or killed by heat (dead bacteria; 1 x 10^8 CFUs/mL: 0% mortality). To make sure the autoclaving process was effective in killing the bacterial cells, 2 nutrient agar plates were streaked with 10 µL of the autoclaved bacterial suspension and incubated at 28°C for 24 hours. As expected, no bacterial growth was observed. The bacterial concentrations were selected to mirror the lethality of the corresponding fungal treatments.

2.4. Preparation of Petri dish nests

Petri dish nests housing experimental mini-colonies of *R. flavipes* were established as described elsewhere (Davis et al., 2018). To form nests, we introduced a total of 49 healthy termites (not including the focal individual) and only three termite castes were use in this study: 46 medium-to-large workers (3-5 mm body length), 2 reproductives and 1 soldier. Humidity and maintenance conditions of the experimental Petri dish nests were as described elsewhere (Chapter I). After 15 days of colony establishment, nests debris was cleaned 24 hours prior to the behavioural experiment and sealed after cleaning was completed.

2.5. Injection of focal termites

We marked focal termites with Nile blue (a fat-soluble stain) so that infected workers could be identified when introduced into the mini colony as described by Davis et al. (Davis et al., 2018). Each focal termite was anaesthetized with CO_2, then injected with 41,4 nL of either viable or dead blastospores, viable or dead bacteria or Ringer´s solution directly into the

hemocoel using the Nanoject II (Drummond Scientific Company, USA). The needle was inserted into the side of the thorax. Using live and heat-killed bacterial and fungal treatments offered potential for both active (immune upregulation through exposure to an infectious individual) and passive (immune upregulation though transfer of immune effectors from conspecifics) form of social immunization (Gallagher et al., 2018; Konrad et al., 2012; Liu et al., 2015; Esparza-Mora et al., 2020). This was also done to be able to infer whether the dead cells are still getting recognized as non-self because of pathogen-associated molecular patterns (PAMPs) found on the surface of bacteria and blastospores (Anjugam et al., 2016). A stronger immune response could be expected towards the dead blastospores and dead bacteria treatments if that is the case. Injected focal termites were kept individually in small Petri dishes (35 mm), each containing a Pall cellulose pad moistened with 1 mL of distilled water and then incubated for 12 hours at 27°C, 70% humidity before use in the behavioural experiment. To study how termites respond to different infection types, we injected focal termites with the following fungal and bacterial treatments or Ringer´s solution: 5 x 10^6 dead blastospores/mL (dead blastospores [0%]), 5 x 10^8 dead blastospores/mL (dead blastospores [40%]) and 5 x 10^8 blastospores/mL (viable blastospores [100%]), representing 0%, 40% and 100% mortality respectively. The following *P. entomophila* doses were selected: 1 x 10^8 (dead bacteria [0%]), 3 x 10^4 (viable bacteria [40%]) and 5 x 10^5 CFUs/mL (viable bacteria [100%]) corresponding to equivalent levels of lethality as the *M. anisopliae* treatments (0%, 40% and 100% mortality, respectively). After injection, all focal termites were incubated individually for 12 hours. Termites injected with the selected intermediate and high doses of bacteria (viable bacteria [40%] and viable bacteria [100%]) were moribund by 14 and 18 hours after injection respectively, with death occurring between 24 and 36 hours (Supplementary Table S1). For individuals injected with dead bacteria (dead bacteria [0%]), symptoms were not observed (Supplementary Table S1). Incubation times were selected based on previous experiments that explored termite collective behavioural responses to infected termites with viable and dead *M. anisopliae* blastospores at different phases of internal infection (Chapter I).

2.6. Behavioural recording

At 12 hours post-injection, infected focal termites were added individually to the Petri dish nests. Petri dishes were not opened or moved after sealing. This process took approximately 15 minutes, and the observation period began immediately after the last dish was sealed. Data was collected by performing scan sampling of each Petri dish nest every 5 minutes for a total of 2 hours (Altmann, 1974). Scanning typically took around 2 minutes. The procedure was repeated with the same focal individuals for the time points 15, 18, 21 and 24 hours post infection, resulting in a repeated measures experimental design. All observations were performed with the same conditions as we previously described. Behavioural categories relevant to social immunity were defined and classified according to Davis et al. (Davis et al., 2018): groomed by n (gb), cannibalized (c), buried (b), other (o) in addition to a further category, eliminated (e): focal termite body has been fully devoured by nestmates. The percentage of focal termites that were eliminated after 36 hours post infection was calculated,

this included partially cannibalized pieces of focal termites that would nevertheless become eliminated.

2.7. Statistical analysis

Statistical analyses were performed in R version 4.0.2 and Rstudio version 1.3.1056.

2.7.1. Grooming

In order to compare the amount of grooming (defined as the number of grooming states/total observed states) in each treatment, we fitted a generalized linear mixed model (GLMM) to the data using the *glmer* function in the R package *lme4* (Bates et al., 2015). A binomial error distribution was used to model proportion data (Crawley, 2015).

Models for each time point (12, 15, 18, 21 and 24 hours) contained treatment, amount of cannibalism (defined as the number of cannibalism states/total observed states) and colony as a fixed effect. Petri dish nest ID ("Plate") was used as a random effect. All models were compared using Akaike information criterion (*AIC*-function in R), which revealed the full model to be the best fit. Model assumptions were checked using the functions *simulateResiduals*, *testDispersion* and *testZeroInflation* contained within the *DHARMa* package (Hartig, 2020). Post hoc comparisons were performed using the *glht* function with Tukey correction from the *multcomp* package v1.4-10 (Korner-Nievergelt et al., 2015a, b).

2.7.2. Survival of termites kept individually

Individual *R. flavipes* termites from three different colonies (11+13, 5 and X) were assigned to one of the following fungal treatments: (i) 60 individuals: Viable blastospores [100%] (injected 5 x 10^8 blastospores/mL); (ii) 60 individuals: Dead blastospores [40%] (injected 5 x 10^8 heat-killed blastospores/mL); (iii) 60 individuals: Dead blastospores [0%] (injected 5 x 10^6 heat-killed blastospores/mL); (iv) 60 individuals: Control (injected Ringer's solution) and bacterial treatments: (i) 60 individuals: Viable bacteria [100%] (injected 5 x 10^5 CFUs/mL); (ii) 60 individuals: Viable bacteria [40%] (injected , 3 x 10^4 CFUs/mL); (iii) 60 individuals: Dead bacteria [0%] (injected 1 x 10^8 heat-killed CFUs/mL); (iv) 60 individuals: Control (injected Ringer's solution). Survival of each individual was recorded every 12 hours for 240 and 96 hours for fungal and bacterial treatments, respectively. The effect of treatment on survival was analysed using Cox proportional hazard models with the package *coxme* (Therneau et al., 2020). Median survival time for each treatment was calculated using the *survminer* package (Kassambara et al., 2017). Control data were right-censored, we uncensored one randomly selected individual from the control treatment per colony, following Tragust et al. (2013). The *glht*-function was used to perform post-hoc pairwise comparisons using Tukey tests with Bonferroni correction, using the package *multcomp* v1.4-10 (Hothorn et al., 2012).

2.7.3. Cannibalism

In order to analyse the effect of the different treatments on the occurrence of cannibalism behaviour the *survfit* function from the survival package was used (Therneau et al., 2020). Survival of each focal individual was recorded after 26 hours. Data were plotted using the *ggsurvplot* function from the *survminer* package (Kassambara and Kosinski, 2017). Survival curves were compared using a mixed effect Cox model (*coxme* function from the *coxme* package (Therneau et al., 2020)) in which treatment was used as a fixed effect and Petri dish nest ID ("Plate") as a random effect. The *glht*-function was used to perform post-hoc pairwise comparisons using Tukey tests with Bonferroni correction, using the package *multcomp* v1.4-10 (Hothorn et al., 2012).

2.7.4. Elimination

Complete elimination of each focal individual was recorded 36 hours after injection using the *survfit* function from the survival package was used (Therneau et al., 2020). Data were plotted using the *ggsurvplot* function from the *survminer* package (Kassambara and Kosinski, 2017). Survival curves were compared using a mixed effect Cox model (*coxme* function from the *coxme* package (Therneau et al., 2020)) in which treatment was used as fixed effect and Petri dish nest ID ("Plate") as a random effect. The *glht*-function was used to perform post-hoc pairwise comparisons using Tukey tests with Bonferroni correction, using the package *multcomp* v1.4-10 (Hothorn et al., 2012).

2.8. CHC analysis

Extractions of pools of three individual termites per treatment were performed in 2 ml glass vials (Agilent Technologies, Santa Clara, California, USA) on an orbital shaker (IKA KS 130 Basic, Staufen, Germany) for 10 minutes. Extracts were subsequently evaporated under a constant stream of gaseous carbon dioxide and then resuspended in 5 µL in a hexane solution containing 7.5 ng/µL dodecane (C12) as an internal standard. Following this, 3 µL of the resuspended extracts were injected in splitless mode with an automatic liquid sampler (ALS) (PAL RSI 120, CTC Analytics AG, Switzerland) into a gas-chromatograph (GC: 7890B) simultaneously coupled to a flame ionization detector (FID: G3440B) and a tandem mass spectrometer (MS/MS: 7010B, all provided by Agilent Technologies, Waldbronn, Germany). The system was equipped with a fused silica column (DB-5MS ultra inert; 30 m x 250 µm x 0.25 µm; Agilent J&W GC columns, Santa Clara, CA, USA) at a temperature of 300 °C with helium used as a carrier gas under a constant flow of 1.8 mL/min. The FID had a temperature of 300 °C and used nitrogen with a 20 mL/min flow rate as make-up gas and hydrogen with a 30 mL/min flow rate as fuel gas. The column was split at an auxiliary electronic pressure control (Aux EPC) module into an additional deactivated fused silica column piece (0.9 m x 250 µm x 0.25 µm) with a flow rate of 0.8 mL/min leading into the FID detector, and another deactivated fused silica column piece (1.33 m x 250 µm x 0.25 µm) at a flow rate of 1.33 mL/min into the

mass spectrometer. The column temperature program started at 60 °C and was held for 5 min, increasing 20 °C/min up to 200 °C and then increasing 3 °C/min to the final temperature of 325 °C, held for 5 min.

CHC peak detection, integration, quantification and identification were all carried out with Quantitative Analysis MassHunter Workstation Software (Version B.09.00 / Build 9.0.647.0, Agilent Technologies, Santa Clara, California, USA). CHCs were identified according to their retention indices, diagnostic ions, and mass spectra as provided by the total ion count (TIC) chromatograms, whereas their quantifications were achieved by the simultaneously obtained FID chromatograms, allowing for the best-suited method for hydrocarbon quantification (Agilent Technologies, Waldbronn, Germany, pers. comm.) while simultaneously retaining the capability to reliably identify each compound. Absolute CHC quantities (in ng) were obtained by calibrating each compound according to a dilution series based on the closest eluting n-alkane from a C21-40 standard series (Merck, KGaA, Darmstadt, Germany) at 0.5, 1, 5, 10, 20, 40 ng/µl, respectively. A discriminant analysis (DA) was performed with the R package "MASS" to test whether CHC profiles statistically differ between the investigated treatment groups and to visualize the degree of separation between them.

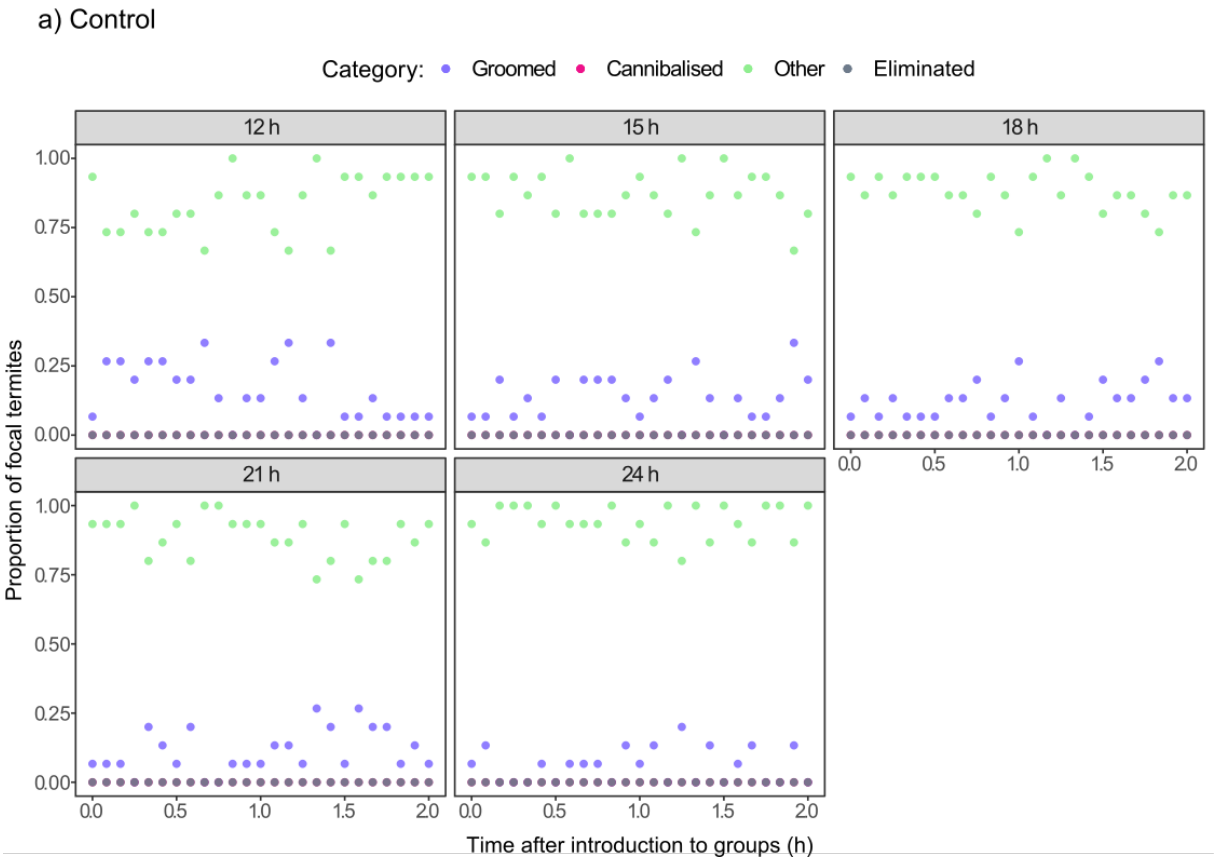
3. Results

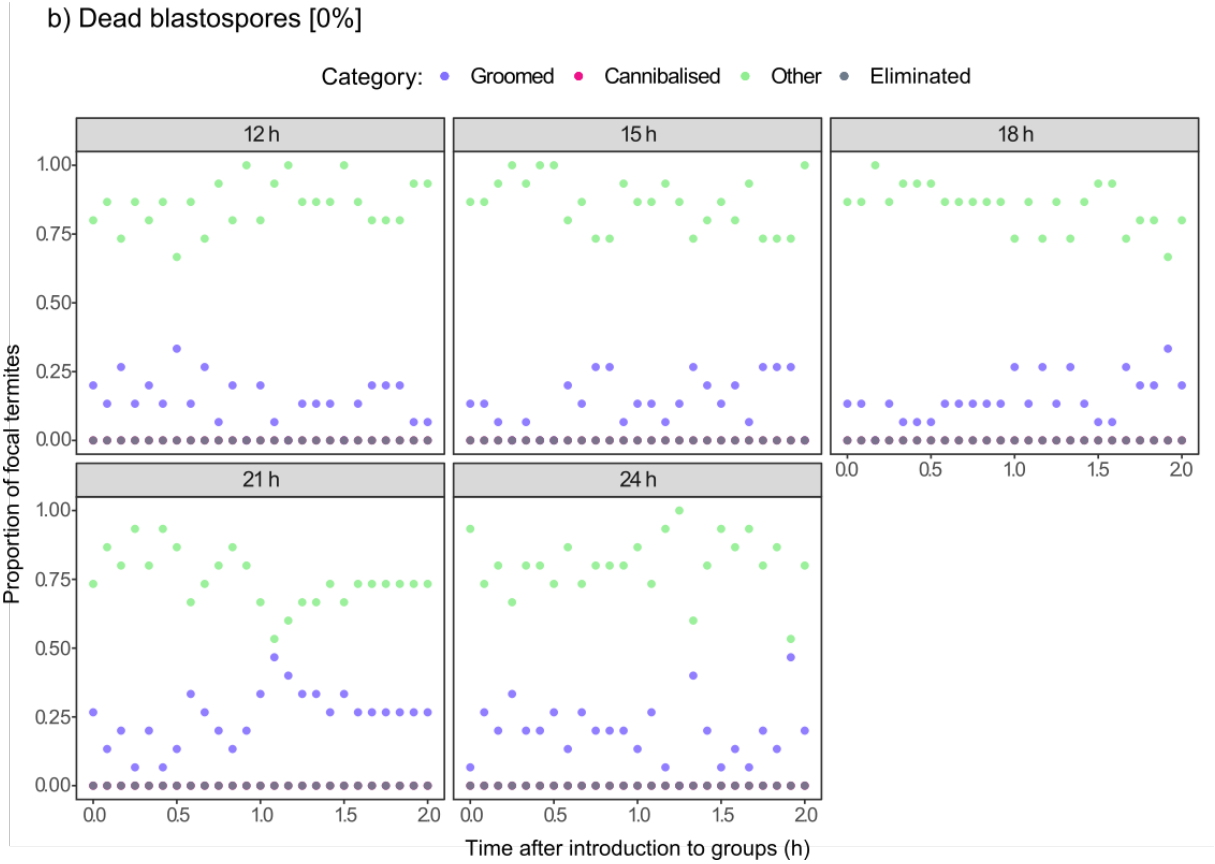
3.1. Overview of social immune responses

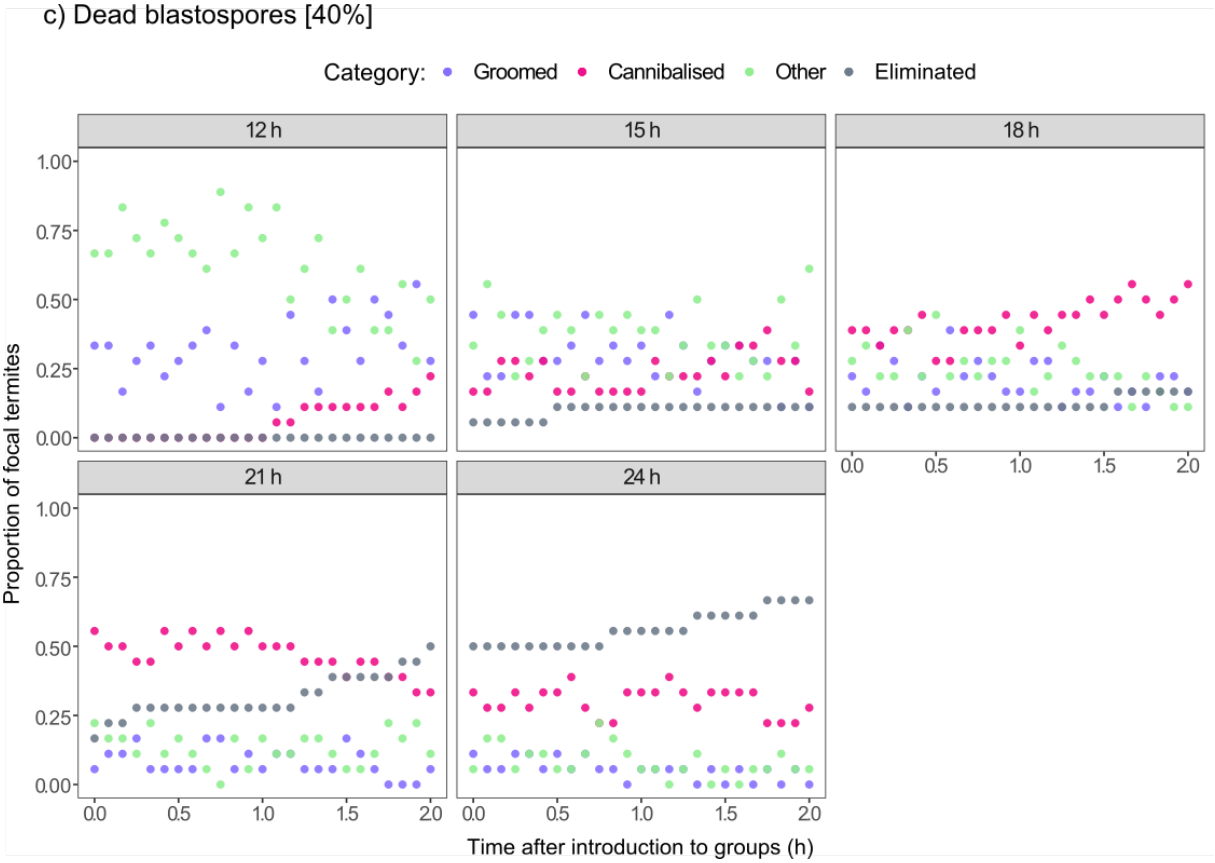
We focused on 5 behavioural categories: grooming, cannibalism, "other" (states unrelated to social immunity, e.g. walking), burial and eliminated. Burial data were not analyzed as this behaviour was not recorded. Focal termites were visible throughout all experiment, and there was one case of a focal termite cadaver being ignored.

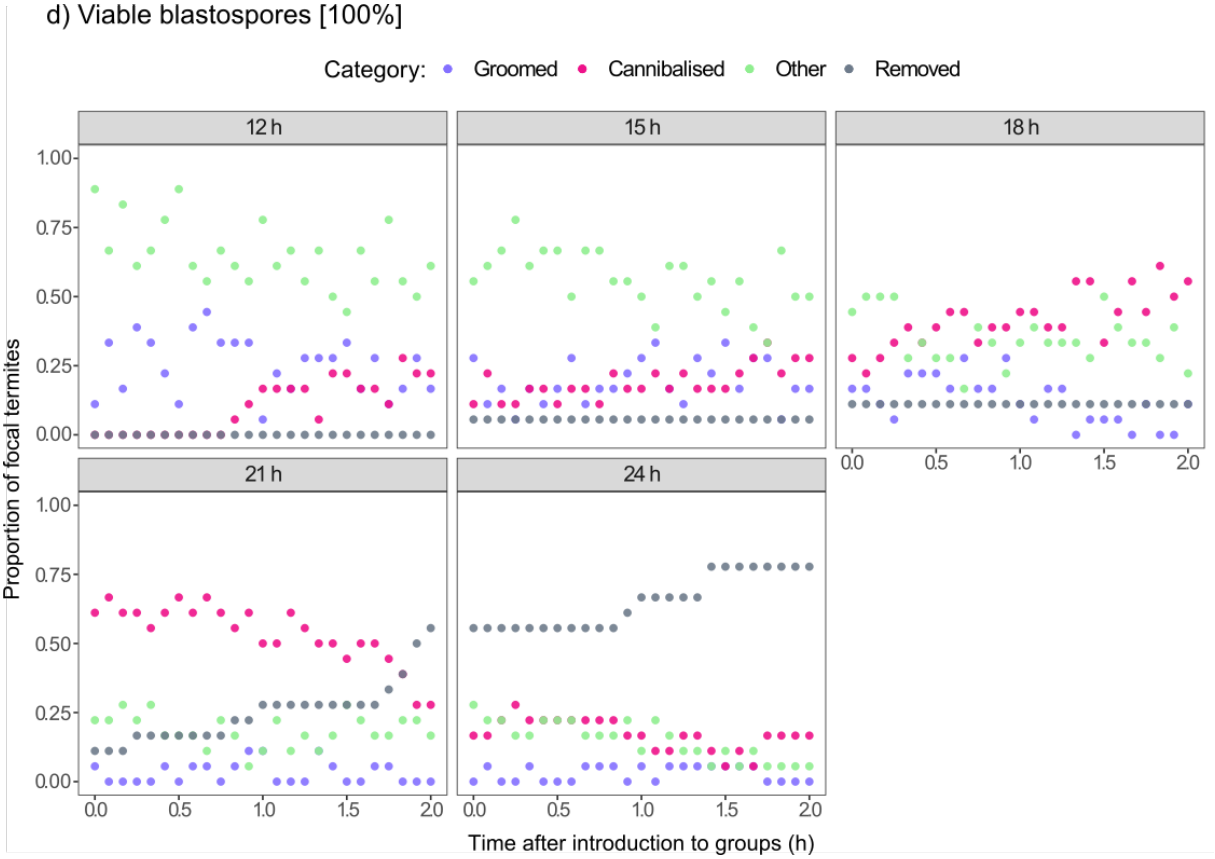
The behavioural repertoire of control-treated termites (Ringer, dead blastospores [0%], dead bacteria [0%]) did not vary significantly over the entire course of the experiment, with most individuals falling under the "other" category (**Figure 1a, b, e**), low levels of grooming and not a single individual getting cannibalized or subsequently eliminated except for isolated recorded cases of cannibalism in the dead bacteria [0%] treatment in the latter time points (21 and 24 hours). By contrast, the pattern was very dynamic for the lethal fungal (dead blastospores [40%], viable blastospores [100%]) and bacterial treatments (viable bacteria [40%], viable bacteria [100%]) (**Figure 1c, d, f, g**). While in the beginning of the observation period (12 hours) the response was similar to that of the control treatments (individuals mostly exhibiting behaviours falling under the "other" category), there was a gradual yet prominent shift to grooming and subsequently cannibalism by 12 hours. In the viable bacteria [100%] and viable blastospore [100%] treatments grooming was increasingly replaced by cannibalism by 15 hours post-injection. By the end of the observation period, a considerable proportion of focal termites in the dead blastospores [40%], viable blastospores [100%] and viable bacteria [100%] treatments had been completely eliminated. In contrast, focal termites

treated with viable bacteria [40%] were not removed by the end of the observation period in the last time point (24 hours).

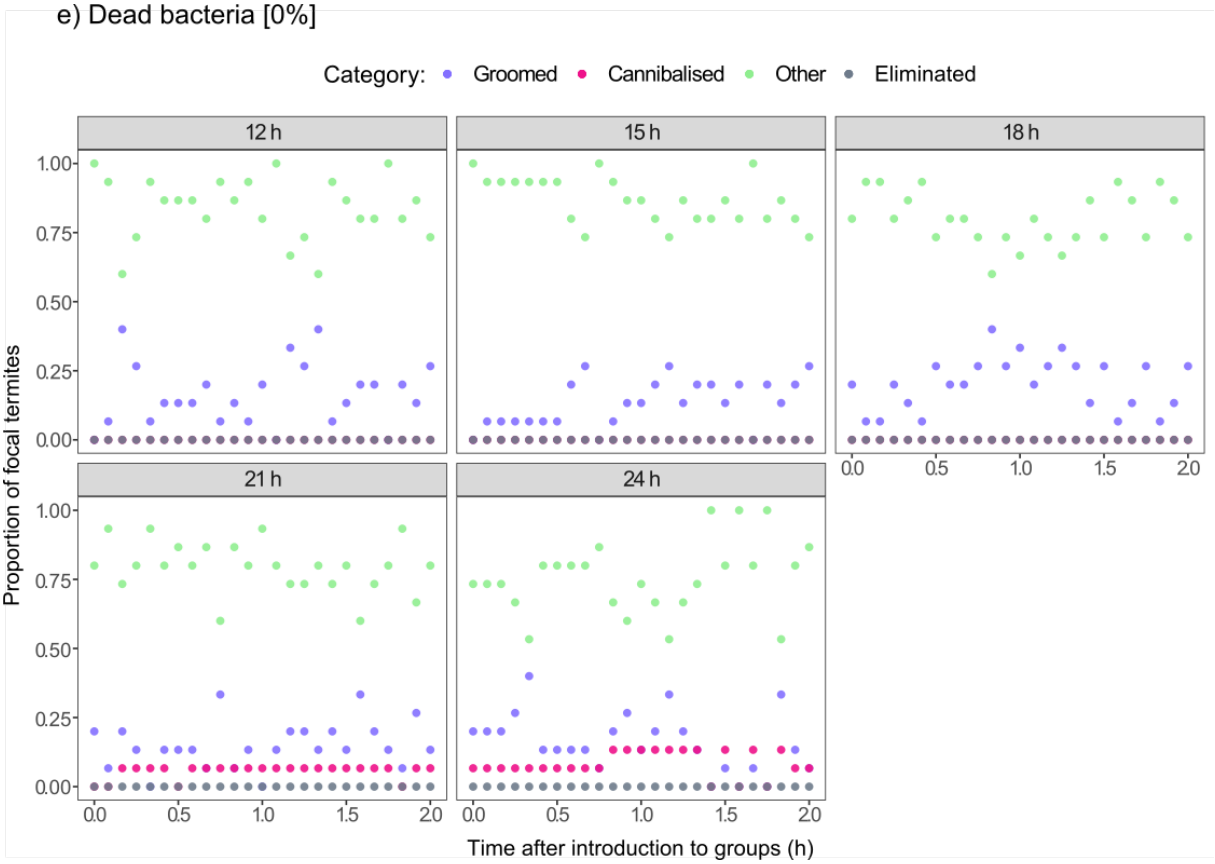




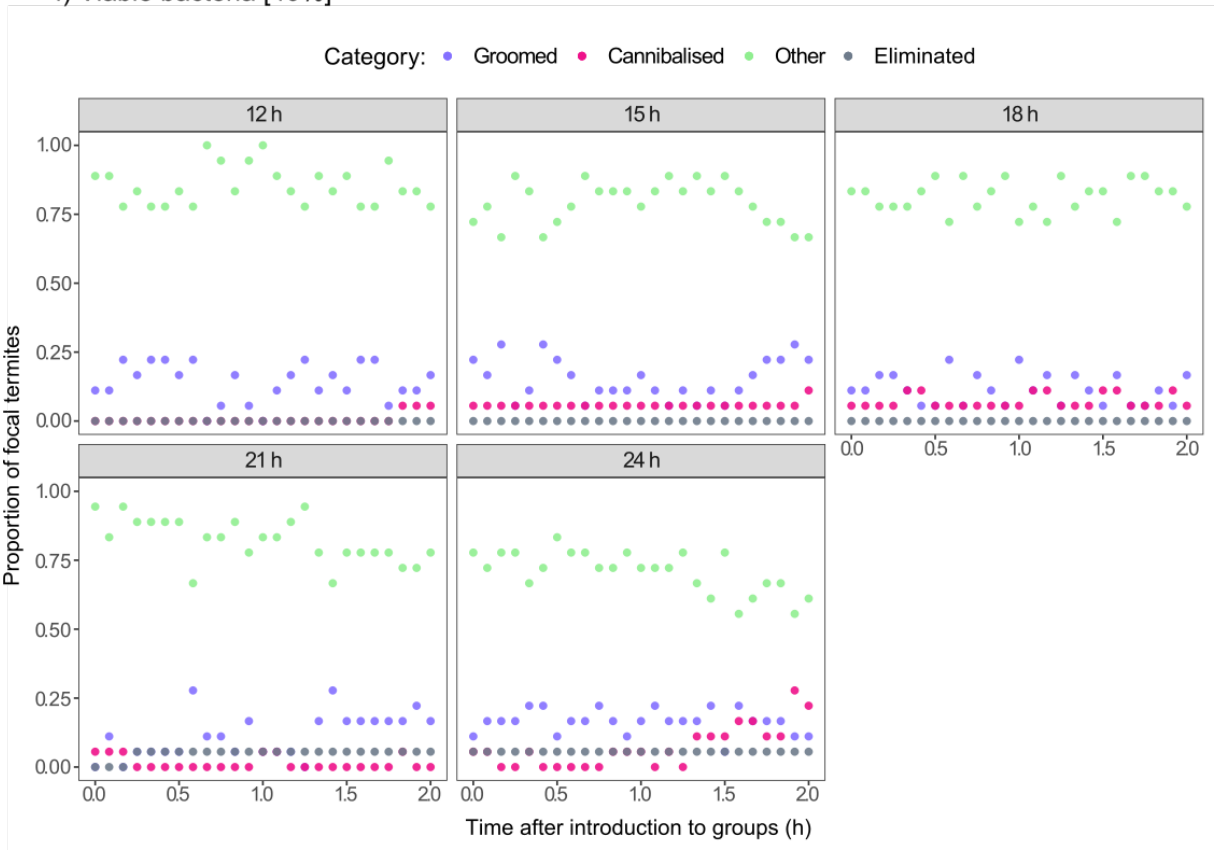

c) Dead blastospores [40%]
Category: Groomed Cannibalised Other Eliminated
12 h
15 h
18 h
21 h
24 h
Proportion of focal termites
Time after introduction to groups (h)


d) Viable blastospores [100%]
Category: Groomed Cannibalised Other Removed
12 h
15 h
18 h
21 h
24 h
Proportion of focal termites
Time after introduction to groups (h)

e) Dead bacteria [0%]



f) Viable bacteria [40%]



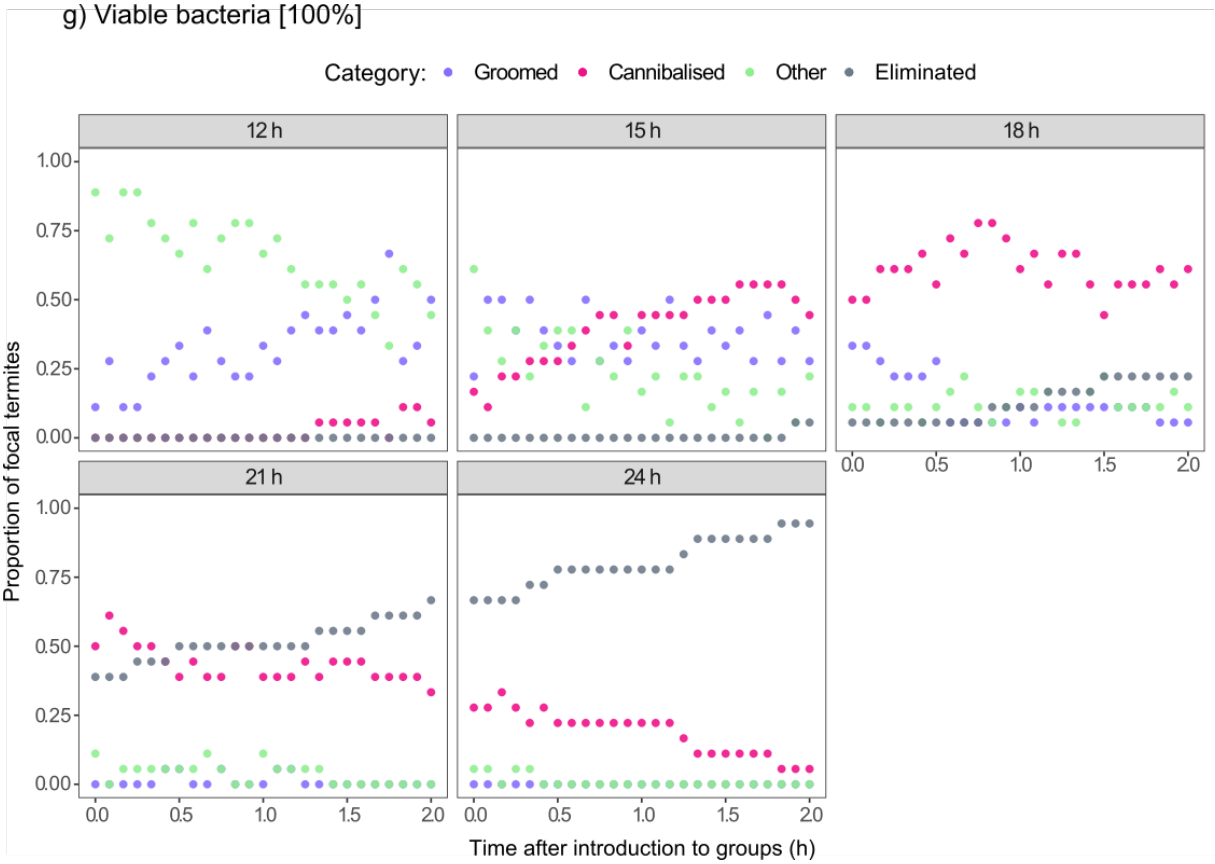


Figure 1. The transition of different behaviours over time during the 2 hours observation period for each of the treatments a) Control (Ringer), b) dead blastospores [0%], c) dead blastospores [40%], d) viable blastospores [100%], e) dead bacteria [0%], f) viable bacteria [40%], g) viable bacteria [100%]. Each point represents the proportion of focal termites that were observed in a given state during the scan.

3.2. Allogrooming response depends on pathogen type and intensity of challenge

The amount of grooming was significantly higher in the dead blastospores [40%] as well viable bacteria [100%] treatments in the first time points (12 and 15 hours) and decreased with time while for the dead blastospores [0%], dead bacteria [0%] and control treatments, it remained almost unchanged, with no significant differences detected between any of the time points. At 12 hours, grooming was significantly elevated when injected with dead blastospores [40%] and viable bacteria [100%] compared with the control (dead blastospores [40%] vs Ringer z= 3.153 *P*= 0.03398; dead blastospores [40%] vs dead blastospores [0%] z = 3.564 *P* = 0.00766; dead blastospores [40%] vs dead bacteria [0%] z = -3.163 *P* = 0.03277; viable bacteria [100%] vs Ringer z = 3.499 *P* = 0.00982; viable bacteria [100%] vs dead blastospores [0%] z = 3.885 *P* = 0.00215; viable bacteria [100%] vs dead bacteria [0%] z = 3.515 *P* = 0.00923). Grooming was also significantly increased in response to viable bacteria [100%] treated focal termites compared with viable bacteria [40%] (z = 4.281 *P* = <0.001) as was also the case in response to dead blastospore [40%] treated focal termites versus viable bacteria [40%] (z = -3.938 *P* = 0.00173) (**Figure 2, Supplementary Table S2**). Also, an interesting and significant difference was obtained between dead blastospores [40%] and viable bacteria [40%] (z= -3.863 *P* =

0.00235). Grooming in the viable bacteria [100%] treatment was significantly elevated over viable bacteria [40%] and viable blastospores [100%] treatments (viable bacteria [100%] vs viable bacteria [40%] z = 5.865 *P* = <0.001; viable bacteria [100%] vs viable blastospores [100%] z = 4.511 *P* = <0.001) at 15 hours but not at any other stage of infection (**Figure 2, Supplementary Table S2**). Significant differences in the grooming response were not found in any of the treatments at 18 hours. The proportion of grooming decreased progressively over time, and this corresponded with an increasingly higher proportion of cannibalism for the time points 15, 18 and 21 hours as well as with a high number of challenged-termites already being eliminated (fully devoured by nestmates) by 24 hours post injection (**Figure 3, Supplementary Table S3, Figures S2, S3**). No significant differences in grooming responses were detected between dead blastospores [40%], viable bacteria [40%], viable blastospores [100%] and viable bacteria [100%] treated focal termites at 21 hours. Amount of grooming was significantly higher for the control treatments at 21 hours (viable bacteria [100%] vs Ringer z = -3.071 *P* = 0.0448; viable bacteria [100%] vs dead blastospores [0%] z = -4.485 *P* = <0.01; viable bacteria [100%] vs dead bacteria [0%] z = -3.665 *P* = 0.0052; viable blastospores [100%] vs dead blastospores [0%] z = -3.604 *P* = 0.0066). Grooming was completely replaced by cannibalism in the dead blastospores [40%], viable blastospores [100%] and viable bacteria [100%] at 21 and 24 hours (**Figure 2, Supplementary Table S2, Figure S1**).

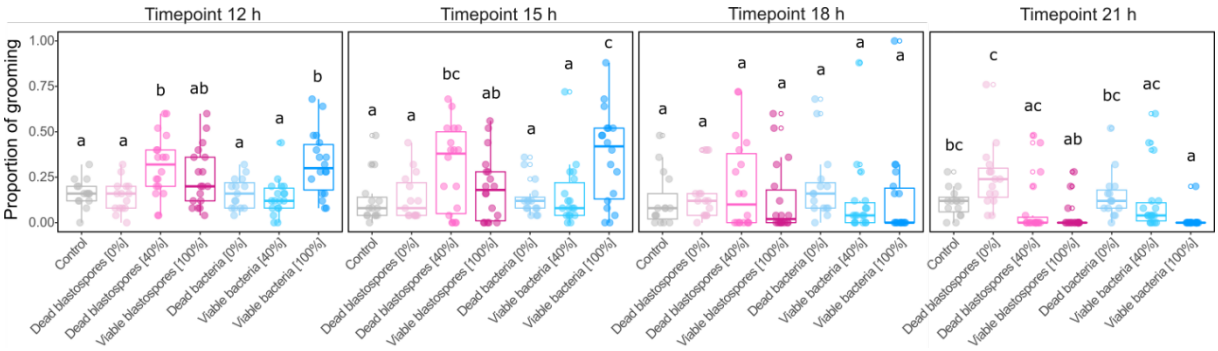


Figure 2. Grooming as a proportion of total visible states over time for every treatment (marked with different colors). Treatments marked with different letters were significantly different from each other. Lower and upper hinges indicate first and third quartiles, respectively. The bold middle line represents the median. Whiskers extend to the smallest/largest value if not smaller/greater than 1.5 times the interquartile range (box length).

3.3. Effect of fungal and bacterial injection on individual termite mortality

Significant differences in survival of termites kept individually were observed within fungal (**Figure 3a**) and bacterial treatments (**Figure 3b**). Survival following injection with dead blastospores [40%], viable blastospores [100%] were significantly different from Ringer and dead blastospores [0%] (Dead blastospores [40%] vs Ringer z = 3.523 *P* = 0.002562; dead blastospores [40%] vs dead blastospores [0%] z = 3.928 *P* = <0.0001; viable blastospores [100%] vs Ringer z = 6.068 *P* <0.0001; viable blastospores [100%] vs dead blastospores [0%] z = 7.357 *P* < 0.0001). Significant differences were also found between viable blastospores [100%] vs dead blastospores [40%] treatments (z = 8.477 *P* <0.0001). On the other hand,

survival of termites kept individually that were injected with similar doses of viable bacteria [100%] and viable bacteria [40%] were significantly different from Ringer and dead bacteria [0%] (viable bacteria [40%] vs Ringer z = 3.633 *P* = 0.00168; viable bacteria [40%] vs dead bacteria [0%] z = 3.631 *P* = 0.00169; viable bacteria [100%] vs Ringer z = 5.354 *P* <0.0001; viable bacteria [100%] vs dead bacteria [0%] z = 5.352 *P* <0.0001). Significant differences were also found between viable bacteria [100%] vs viable bacteria [40%] treatments (z = -6.788 *P* <0.0001).

3.4. Increased elimination behaviours against fungal over bacterial challenge

Focal termites treated with viable blastospores [100%], viable bacteria [100%] and dead blastospores [40%], exhibited largely reduced survival. The amount of observed cannibalism and therefore overall survival probability did not differ between these treatments (**Figure 3c, Supplementary Figure S1, Table S3).** Significant differences were found upon comparison between these three treatments and control, dead blastospores [0%], dead bacteria [0%] treatments (dead blastospores [40%] vs Ringer z = 4.202 *P* = <0.001; dead blastospores [40%] vs dead blastospores [0%] z = 4.202 *P* = <0.001; dead blastospores [40%] vs dead bacteria [0%] z = -4.641 *P* = <0.001; viable blastospores [100%] vs Ringer z = 4.100 *P* = <0.001; viable blastospores [100%] vs dead blastospores [0%] z = 4.100 *P* = <0.001; viable blastospores [100%] vs dead bacteria [0%] z = -4.512*P* = <0.001; viable bacteria [100%] vs Ringer z = 4.621 *P* = <0.001; viable bacteria [100%] vs dead blastospores [0%] z = 4.621 *P* = <0.001; viable bacteria [100%] vs dead bacteria [0%] z = 5.168 *P* = <0.001). One focal termite in the dead blastospore [0%] and two in the dead bacteria [0%] treatments, did not survive for the entire length of the experiment. An interesting difference was observed when comparing the dead blastospore [40%] with the viable bacteria [40%] treatment, with significantly higher amounts of cannibalism being observed in the latter (z = -4.701 *P* = <0.001). Survival differences were also detected between viable blastospores [100%] versus viable bacteria [40%] (z = -4.523 *P* = <0.001) and viable bacteria [100%] versus viable bacteria [40%] treatments (z = 5.417 *P* = <0.001) (**Figure 3c**). Although no differences in survival were found between the viable blastospores [100%] and viable bacteria [100%] treatments, cannibalism appeared to occur earlier in termites infected with viable blastospores [100%] than with viable bacteria [100%].

Termites kept individually that were injected with viable bacteria [100%] died faster than termites injected with viable blastospores [100%] (**Figure 3a, b**). In contrast, a majority of termites injected with dead blastospores [40%] or an equal dose of viable bacteria [40%] survived (**Figure 3a, b**). But when focal termites injected with viable bacteria [40%] were introduced into experimental colonies, these were not cannibalized as much as with dead blastospores [40%] by 24 hours after introduction (**Figure 3c**).

Elimination of focal termites 36 hours after injection with viable blastospores [100%], viable bacteria [100%] and dead blastospores [40%] treatments was significantly different from the controls, dead blastospores [0%] and dead bacteria [0%] treatments (dead blastospores [40%]

vs Ringer z = 3.909 P = 0.00138; dead blastospores [40%] vs dead blastospores [0%] z = 4.392 *P* = <0.001; dead blastospores [40%] vs dead bacteria [0%] z = -4.392 *P* = <0.001; viable blastospores [100%] vs Ringer z = 4.045 *P* = <0.001; viable blastospores [100%] vs dead blastospores [0%] z = 4.571 *P* = <0.001; viable blastospores [100%] vs dead bacteria [0%] z = -4.571 *P* = <0.001; viable bacteria [100%] vs Ringer z = 4.558 *P* = <0.001; viable bacteria [100%] vs dead blastospores [0%] z = 5.249 *P* = <0.001; viable bacteria [100%] vs dead bacteria [0%] z = 5.249 *P* = <0.001). However, differences in elimination of focal termites infected with viable blastospores [100%], viable bacteria [100%] and dead blastospores [40%] were not detected (**Supplementary Table S4, Figure S2, S3**). There was only one case corresponding to viable blastospores [100%] in which the remaining piece of focal termite cadaver was buried.

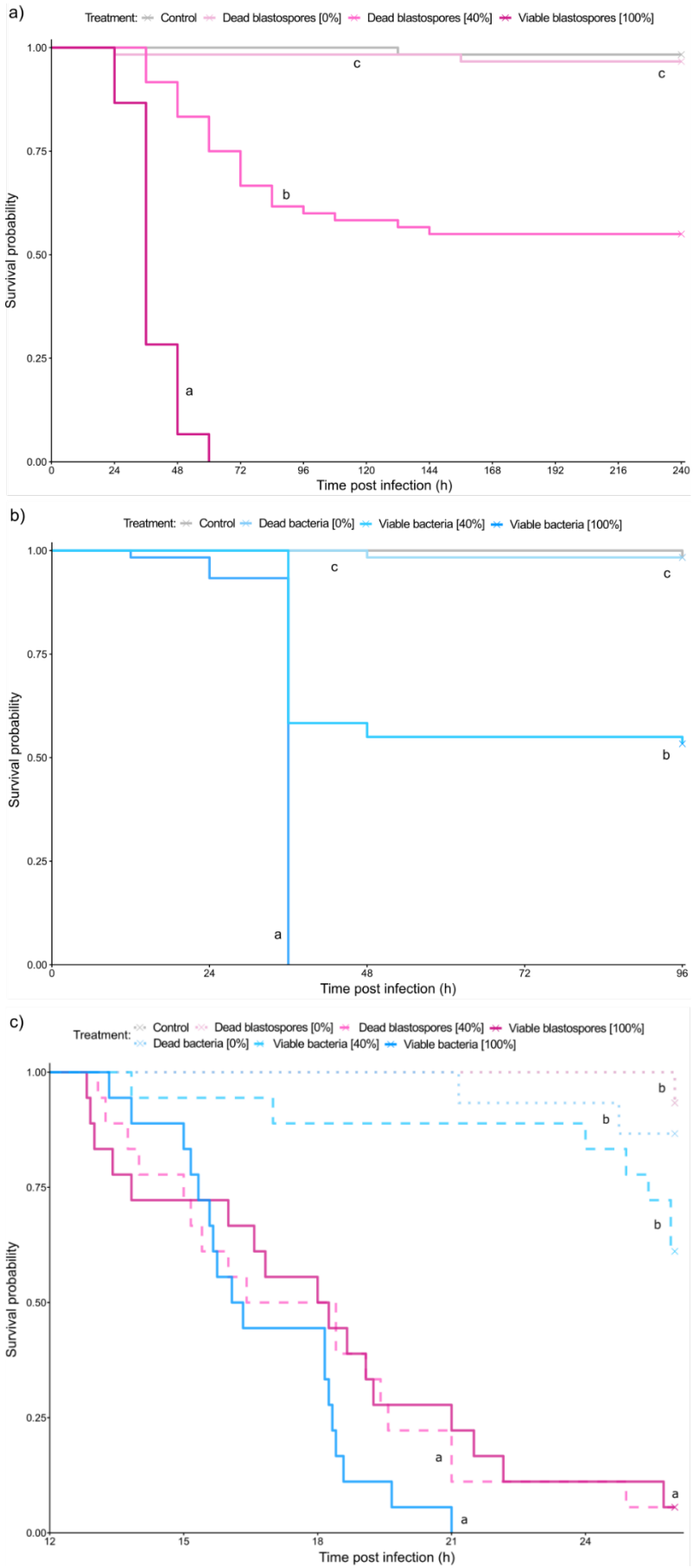


Figure 3. Survival of focal termites kept in isolation (a, b) and following introduction into experimental colonies (c). Survival analysis of the different treatment groups represented by different color

intensities. a) Survival of individual termites injected with different doses of fungal blastospores when kept in isolation. Data from Chapter I. b) Survival of individual termites injected with different doses of bacteria. c) Survival analysis of different treatments, as represented by different colors, following introduction into experimental colonies. For all panels, the x-axis indicates time post injection (in hours) while the y axis depicts survival probability, calculated as the proportion of focal termites from every treatment group that were not cannibalised. Crosses indicate the presence of right-censored data (i.e. focal termites that were not cannibalised during the experiment). Treatments marked with different letters are significantly differently to each other in post hoc comparisons. The control curve in panel c is not visible due to overlap with the upper two lines (dead blastospores [0%] and dead bacteria [0%]).

3.5. Cuticular hydrocarbon analysis

A total of 73 individual equivalents of CHC extracts from worker pools (3 individuals each) taken from 3 different *R. flavipes* colonies were pre-defined into five groups for a discriminant analysis (DA) according to the different treatments: 15 hours after injection of dead blastospores [40%], viable blastospores [100%], viable bacteria [40%], viable bacteria [100%] and Ringer´s solution as a control. Overall differentiation of the CHC profiles was significant according to the different treatments (Wilk's $\lambda < 0.05$, $P < 0.05$). Discriminant function 1 accounted for 51.02%, discriminant function 2 for 26.19% and discriminant function 3 for 20.07% of the total variation, amounting to 97,28% of total variance (**Figure 4**).

As most chemical messages in social insects are encoded by quantitative shifts of several com pounds (Leonhardt et al., 2016), gas chromatography-mass spectrometry (GC–MS) analysis confirmed that viable blastospores [100%] infected termites have distinct chemical profiles compared to both bacterial treatments ([40%] and [100%]), dead blastospores [40%] and control. Injected termites with dead blastospores [40%] and both bacterial (viable bacteria [40%], viable bacteria [100%]) treatments do not lead to a visible increase in overall quantities of CHC compounds compared (**Figure 4**).

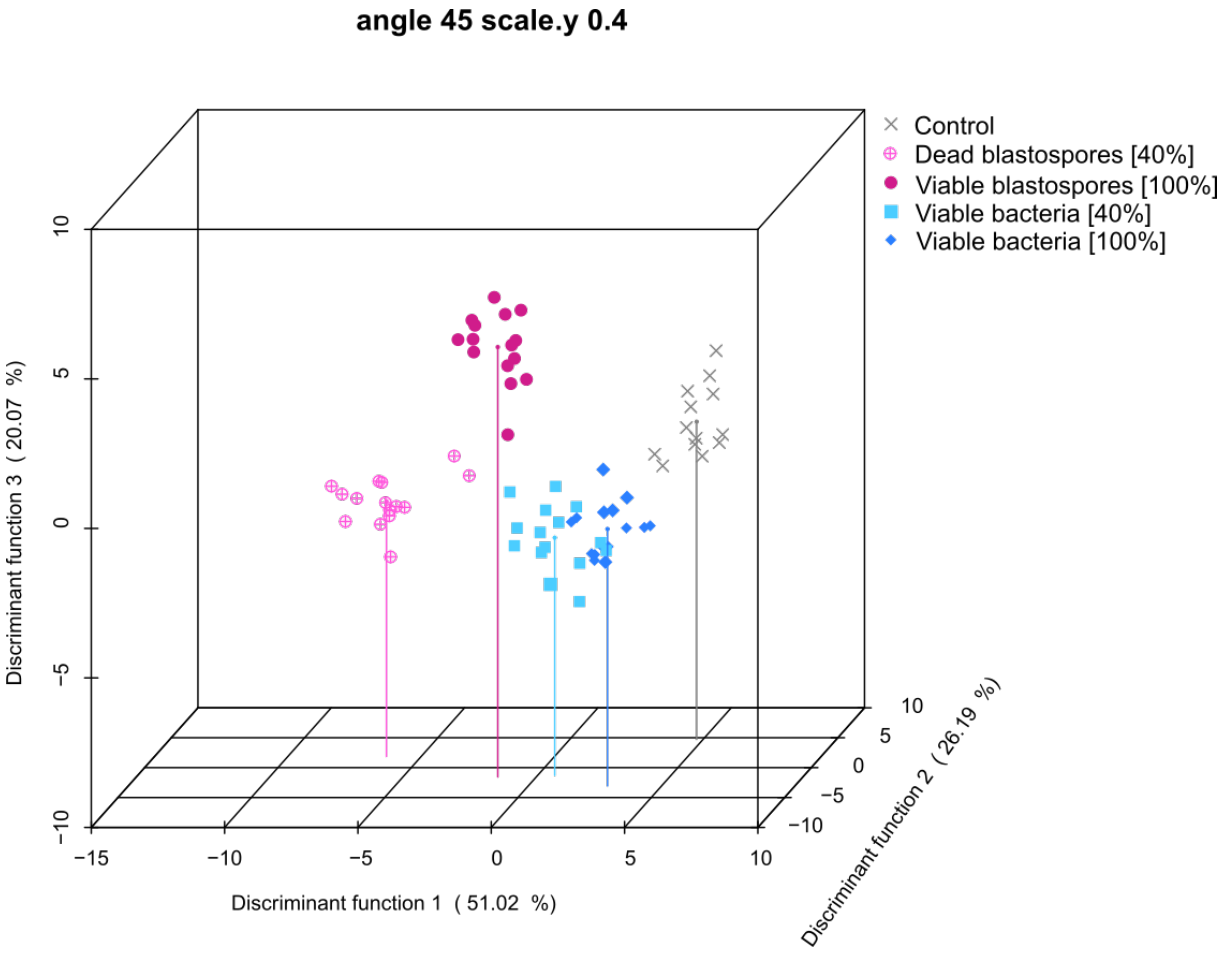


Figure 4. Linear discriminant analysis (LDA) based on 73 cuticular hydrocarbon (CHC) extracts from worker pools (3 individuals each) taken from 3 different *R. flavipes* colonies. The workers were treated with either fungus (dead blastospores [40%], viable blastospores [100%]), bacterial (viable bacteria [40%], viable bacteria [100%]) or Ringer solution as a control, and they were freeze-killed 15 hours after injection. Different colors and symbols indicate each treatment (see legend).

4. Discussion

This study is the first to directly compare termite collective defences in response to two different entomopathogenic challenges – one fungal and one bacterial – of varying intensities. We hypothesized that collective responses, including elimination behaviours mediated by cannibalism, would be greater towards the native fungal (*M. anisopliae*) versus the non-native bacterial (*P. entomophila*) challenge at equivalent severities of infection.

When challenged with a highly lethal dose (100% mortality), focal termites were rapidly eliminated, independent of pathogen type used, indicating that social immune responses are transferable to diverse and potentially novel infection threats like *P. entomophila*. Furthermore, destructive behaviours appeared to overestimate the threat of *M. anisopliae* challenged individuals, as only some bacteria-treated termites but all fungal-treated termites were eliminated by 36 hours p.i. when challenged with an intermediate lethal dose (40% mortality). In line with our prediction, this suggests that elimination behaviours (cannibalism)

are more rapidly deployed against fungal rather than bacterial challenge (at an intermediate challenge), suggesting either that i) the distinct infection modes of bacterial and fungal pathogens or ii) their contrasting evolutionary exposure to termite hosts underlie these differences. In the case of the former possibility, bacterial infections typically occur via oral uptake, so that bacterially-infected individuals will not carry transmissible spores on their cuticle, as is the case with entomopathogenic fungi (Konrad et al., 2012). It also suggests that nestmates may be capable of detecting specific chemical or other triggers associated with blastospore challenge. Elimination as a consistent response to an intermediate 40% mortality-treated individual, regardless of pathogen or viability, further shows that moribundity is not a necessary cue to trigger termite cannibalism (Davis et al., 2018; Chapter I). As the primary kill-component of the care-kill dichotomy of social immunity in termites (Cremer and Sixt, 2009) elimination via cannibalism of infected kin may have evolved to be especially stringent as a pre-emptive defence against transmission. It would be interesting to explore whether similarly over-conservative kill-responses have evolved in other social insect lineages (Pull et al., 2018; Spivak and Gilliam, 1993; Al Toufailia et al., 2018).

With respect to grooming, we observed that grooming of focal termites injected with high doses of viable bacteria was higher than in fungal treatments, but only at the 15-hour timepoint, which corresponded to the earlier onset of cannibalism in response to fungal-treated individuals. Compared with the control treatments, grooming was significantly elevated in fungal 40% mortality-treated individuals at early timepoints, while significantly lower at later timepoints, as a result of the transition to cannibalism during this later time frame. Intriguingly, grooming responses to bacterial 40% mortality-treated individuals did not differ from controls at any observed timepoint, despite the fact that by 36 hours, some individuals of this bacterial treatment were found to be eliminated. Whether there was a similar enhancement of grooming in the time period between 24 and 36 hours remains an open question but given the trend towards increased cannibalism by 24 hours in this treatment, it is plausible that grooming may have been skipped in this case. Since grooming cannot diminish advanced *M. anisopliae* nor bacterial infections because there is nothing to be mechanically removed from the cuticle, it is interesting that such behaviour was nonetheless observed. Whether this represents a behaviourally-mediated inspection phase, perhaps allowing nestmates to detect evidence of stress or immune activation, also remains open. Direct detection of blastospores and bacteria by nestmates seems unlikely, although injection of both could trigger the infected individual to produce chemical signals after activation of the innate immune system. These chemical signals could involve modification of the cuticular hydrocarbon profile. Indeed, we were able to find differential patterns of cuticular hydrocarbon (CHC) profiles among all of the treatments, particularly discernible was the profile associated with the viable blastospores [100%] treatment, which by the investigated time point (15 hours) was associated with the highest observed rates of cannibalism. The exact mechanism is unclear, but we speculate that a mixture of CHCs may accumulate on challenged termite surfaces, eventually reaching a critical threshold that, relative to the other compounds, is sufficient to elicit elimination behaviours.

Similarly, ants may have developed a signalling strategy to indicate signs of infection (Pull et al., 2018). Bacterially challenged (heat-killed and alive) *Tenebrio molitor* also showed differences in immune and fitness traits compared to healthy conspecifics suggesting that *T. molitor* responds to immune-related signals in the social environment, influencing the synthesis of specific hydrocarbons (Richard et al., 2008; 2012) and potentially corresponding to modifications in CHC profiles following immune stimulation (Gallagher et al., 2018). Pathogen-specific signals may be more robust and general (Pull et al., 2018), representing expressions of "find-me and eat-me" signals, akin to infected cells in a body (Cremer, 2019; Conroy & Holman, 2022). Behavioural responses to 40% mortality-injected termites, whilst less prominent than responses to higher doses, suggest that lower level challenge can lead to significant immune stimulation and potentially also trigger changes to cuticular chemistry. For instance, infection with heat-killed bacteria can elicit an immune response, including the increase of antimicrobial peptides (AMPs). This is consistent with Keesey et al., where infection with heat-killed *P. entomophila* resulted in the production of two different AMPs including Drosomycin and Diptericin as well as a smaller but significant increase in fatty-acid-derived pheromone production in *Drosophila* (Keesey et al., 2017). Similarly, Cathepsin D was found to occur in higher amounts in the trophallactic droplets of ants after injection of heat-killed *Serratia marcescens* or lipopolysaccharides (LPS) (Hamilton et al., 2011). Bacterial cell wall components (LPS and peptidoglycan) can be highly immunogenic (Gallagher et al., 2018). In termites, Rosengaus et al., found that injection of killed-bacteria generated an immune response that conferred higher survival to subsequent challenges with live *Pseudomonas aeruginosa* and induction of proteins appeared to differ in bacteria-injected and fungus-exposed termites (Rosengaus et al., 1999b), suggesting specificity in the immune response. In ants, active upregulation of immune gene expression after fungal infection was found to occur, suggesting the induction of a specific immune response distinct from immune responses to bacteria (Konrad et al., 2012). Specific immune upregulation is also known to occur in *Drosophila*. Notably, Gram-negative bacteria have a strong effect in inducing humoral responses compared to Gram-positive bacteria and fungi (Lemaitre et al., 1997). How activation of immune signalling pathways such as those involving Phenoloxidase (PO) (Gillespie et al., 2000), might play an (indirect) role in signalling remains unclear, and it remains uncertain whether wider changes to cuticular chemical biosynthesis are causally associated with immune system activation. It is also possible that termites are responding to more direct cues, including chemicals emitted by replicating pathogens inside the host, although this seems less likely given the strong responses observed towards inactivated blastospore-treated termites. A further critical area of research is the need to empirically test the causal role played by CHCs in triggering social immune responses, for which detailed chemical ecology experiments with individual compounds or blends, are required.

Close contact between infected and naïve nestmates enhances resistance among naïve individuals, and this form of social immunization has been confirmed in ants (Konrad et al., 2012). They showed that social contact between pathogen-exposed and unexposed nestmates caused low-level infections in unexposed individuals, ultimately leading to

upregulation of anti-fungal defences and an enhanced ability to inhibit pathogen replication (Konrad et al., 2012). Such immunization by low-level infections, induces immune gene expression and hence protective immune stimulation, and this has also been shown in termites exposed to *M. anisopliae*. This immune strategy allows nestmates to experience infections that are simply sublethal by actively upregulating their immune system (Liu et al., 2015). This leads to the idea that termites interact with bacterial or fungal challenged nestmates through allogrooming to upregulate their immune system, thereby forming a "protective wall" to prevent pathogen transmission (Liu et al., 2015) as well as facilitating individual immune protection. Such inoculation with low doses of pathogen might therefore enhance resistance to pathogens in eusocial insects (Ezenwa et al., 2016).

Pseudomonas entomophila infections cause insects to rapidly succumb despite the induction of innate immune responses, indicating that this bacterium has developed specific strategies to escape the insect immune response (Lieh et al., 2006). Like *P. entomophila*, *M. anisopliae* produces toxins or virulence factors during host infection (Iwanicki et al., 2020; Tetreau, 2018). They are known to interact with the host immune system in order to suppress the immune response and/or to damage host cells, which in consequence could trigger the release of danger signals that can stimulate an immune response to infection (Tetreau, 2018; Moreno-García et al., 2014). We have previously confirmed that *M. anisopliae* produces destruxins during the infection process in termites and that injection of heat-killed blastospores filtrate contains subcellular components (Chapter I). How social immunity is modulated by specific pathogen toxins also represents a promising avenue for future research. A potential hypothesis to test here is that specific virulence factors associated with co- and non-evolved pathogens may have resulted in significantly different induced individual as well as social immunity impacts. A possibility for experimentally exploring the role of toxins in the regulation of social immune response would be to compare behaviours toward a pathogenic but non-toxic bacterium, or by genetically manipulating known toxin genes to explore their functional role in host immunity at individual and social levels.

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Supplementary material

Sickness cues Experiment

To determine the optimal phase for testing the effect of different infection types with *M. anisopliae* and *P. entomophila* on social immune behaviours, 5 *Reticulitermes flavipes* workers were anaesthetized with CO_2, then injected with 41,4 nL of a suspension containing either viable *Pseudomonas entomophila* 3 x 10^4 (viable bacteria [40%]) or 5 x 10^5 CFUs/mL (viable bacteria [100%]) directly into the hemocoel using the Nanoject II, the needle was inserted into the side of the thorax. Infected termites were kept individually in small Petri dishes (35 mm), each containing a Pall cellulose pad moistened with 1 mL of distilled water and then incubated in darkness for a total of 48 hours at 27°C. Infected termites were checked carefully within 36 hours to determine the symptoms of the infection.

Supplementary Table S1. Description of the symptoms caused by *P. entomophila* injected directly into the termite hemocoel. Different colors (light blue, pink, green and yellow) represent varying signs of disease observed after injection of blastospores. “Signs of weakness”; “Very sick/moribund”; “close to death” refer to the following behaviours, respectively: slower walking; hardly able to walk; antennal movements only or legs if lying dorsally.

Incubation time	**Description**		
	Control	viable bacteria [40%]	viable bacteria [100%]
0 h	No signs of infection	No signs of infection	No signs of infection
2 h	No signs of infection	No signs of infection	No signs of infection
12 h	No signs of infection	No signs of infection	No signs of infection
14 h	No signs of infection	No signs of infection	First signs of weakness
16 h	No signs of infection	No signs of infection	Sick and sluggish
18 h	No signs of infection	First signs of weakness	Sick and sluggish
20 h	No signs of infection	Sick and sluggish	Very sick / moribund
22 h	No signs of infection	Sick and sluggish	Close to death
24 h	No signs of infection	Very sick / moribund	Termites start dying
36 h	No signs of infection	Termites are dead	

Supplementary Table S2. Z and p values from post hoc pairwise comparisons (Tukey tests using a Bonferroni correction) of the proportion of grooming over all observed behaviours. Statistically significant differences are indicated in bold. Empty squares mean that the comparison has already been made and can be found somewhere else in the table.

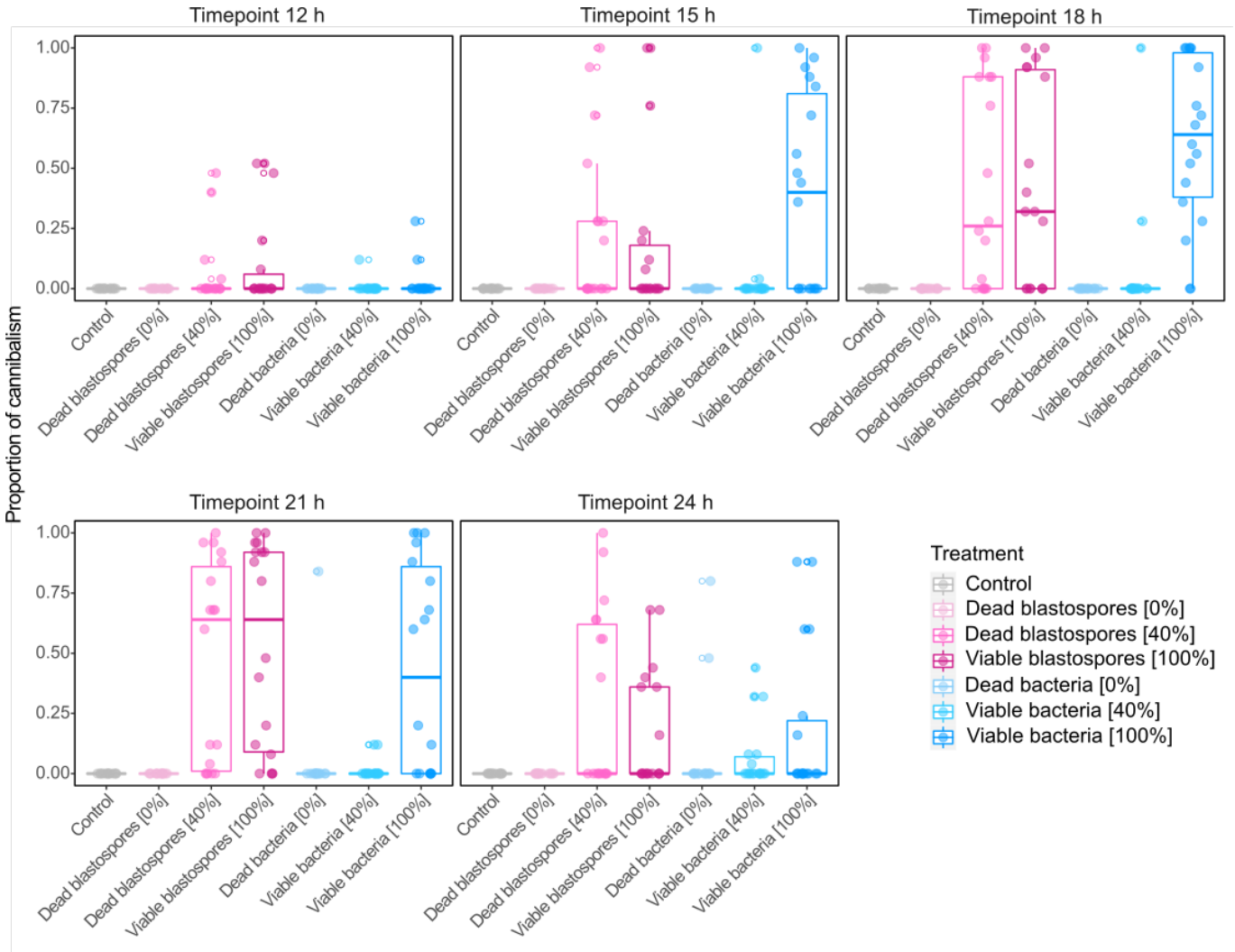
Time point 12 h							
Treatment	Control (Ringer)	0% Blastospores-	40% Blastospores-	100% Blastospores+	0% P. entomophila-	40% P. entomophila	100% P. entomophila
Control (Ringer)		z = -0.559 *P* = 1.00000	**z = 3.153** ***P* = 0.03398**	z = 1.991 *P* = 0.97576	z = 0.062 *P* = 1.00000	z = -0.606 *P* = 1.00000	**z = 3.499** ***P* = 0.00982**
0% Blastospores-			**z = 3.564** ***P* = 0.00766**	z = 2.479 *P* = 0.27658	z = 0.626 *P* = 1.00000	z = -0.009 *P* = 1.00000	**z = 3.885** ***P* = 0.00215**
40% Blastospores-				z = -1.413 *P* = 1.00000	**z = -3.163** ***P* = 0.03277**	**z = -3.938** ***P* = 0.00173**	z = 0.418 *P* = 1.00000
100% Blastospores+					z = -1.974 *P* = 1.00000	z = -2.741 *P* = 0.12871	z = 1.778 *P* = 1.00000
0% P. entomophila-						z = -0.681 *P* = 1.00000	**z = 3.515** ***P* = 0.00923**
40% P. entomophila							**z = 4.281** ***P* = 0.00039**
100% P. entomophila							
Time point 15 h							
Treatment	Control (Ringer)	0% Blastospores-	40% Blastospores-	100% Blastospores+	0% P. entomophila-	40% P. entomophila	100% P. entomophila
Control (Ringer)		z = 0.240 *P* = 1.000000	**z = 4.271** ***P* = 0.000408**	z = 2.170 *P* = 0.630131	z = 0.307 *P* = 1.000000	z = 0.612 *P* = 1.000000	**z = 6.143** ***P* = 1.70e-08**
0% Blastospores-			**z = 4.054** ***P* = 0.001056**	z = 1.936 *P* = 1.000000	z = 0.066 *P* = 1.000000	z = 0.365 *P* = 1.000000	**z = 5.952** ***P* = 5.57e-08**
40% Blastospores-				z = -2.262 *P* = 0.497008	**z = -4.008** ***P* = 0.001289**	**z = -3.863** ***P* = 0.002349**	z = 2.481 *P* = 0.274843
100% Blastospores+					z = -1.876 *P* = 1.000000	z = -1.642 *P* = 1.000000	**z = 4.511** ***P* = 0.000136**
0% P. entomophila-						z = 0.298 *P* = 1.000000	**z = 5.923** ***P* = 6.63e-08**
40% P. entomophila							**z = 5.865** ***P* = 9.44e-08**

100% P. entomophila							
Time point 18 h							
Treatment	Control (Ringer)	0% Blastospores-	40% Blastospores-	100% Blastospores+	0% P. entomophila-	40% P. entomophila	100% P. entomophila
Control (Ringer)		z = 0.881 *P* = 1.000	z = 2.679 *P* = 0.155	z = 1.509 *P* = 1.000	z = 1.471 *P* = 1.000	z = -0.056 *P* = 1.000	z = 2.690 *P* = 0.150
0% Blastospores-			z = 1.902 *P* = 1.000	z = 0.722 *P* = 1.000	z = 0.601 *P* = 1.000	z = -0.960 *P* = 1.000	z = 2.013 *P* = 0.926
40% Blastospores-				z = -1.199 *P* = 1.000	z = -1.359 *P* = 1.000	z = -2.816 *P* = 0.102	z = 0.420 *P* = 1.000
100% Blastospores+					z = -0.184 *P* = 1.0000	z = -1.606 *P* = 1.000	z = 1.521 *P* = 1.000
0% P. entomophila-						z = -1.565 *P* = 1.000	z = 1.543 *P* = 1.000
40% P. entomophila							z = 2.818 *P* = 0.102
100% P. entomophila							
Time point 21 h							
Treatment	Control (Ringer)	0% Blastospores-	40% Blastospores-	100% Blastospores+	0% P. entomophila-	40% P. entomophila	100% P. entomophila
Control (Ringer)		z = 2.053 *P* = 0.841140	z = -0.798 *P* = 1.000000	z = -2.028 *P* = 0.893130	z = 0.794 *P* = 1.000000	z = -0.626 *P* = 1.000000	**z = -3.071** ***P* = 0.044771**
0% Blastospores-			z = -2.498 *P* = 0.261997	**z = -3.604** ***P* = 0.006592**	z = -1.257 *P* = 1.000000	z = -2.722 *P* = 0.136361	**z = -4.485** ***P* = 0.000153**
40% Blastospores-				z = -1.366 *P* = 1.000000	z = 1.496 *P* = 1.000000	z = 0.292 *P* = 1.000000	z = -2.474 *P* = 0.280462
100% Blastospores+					z = 2.695 *P* = 0.147955	z = 1.590 *P* = 1.000000	z = -1.215 *P* = 1.000000
0% P. entomophila-						z = -1.436 *P* = 1.000000	**z = -3.665** ***P* = 0.005191**
40% P. entomophila							z = -2.702 *P* = 0.144850
100% P. entomophila							
Time point 24 h							
Treatment	Control (Ringer)	0% Blastospores-	40% Blastospores-	100% Blastospores+	0% P. entomophila-	40% P. entomophila	100% P. entomophila

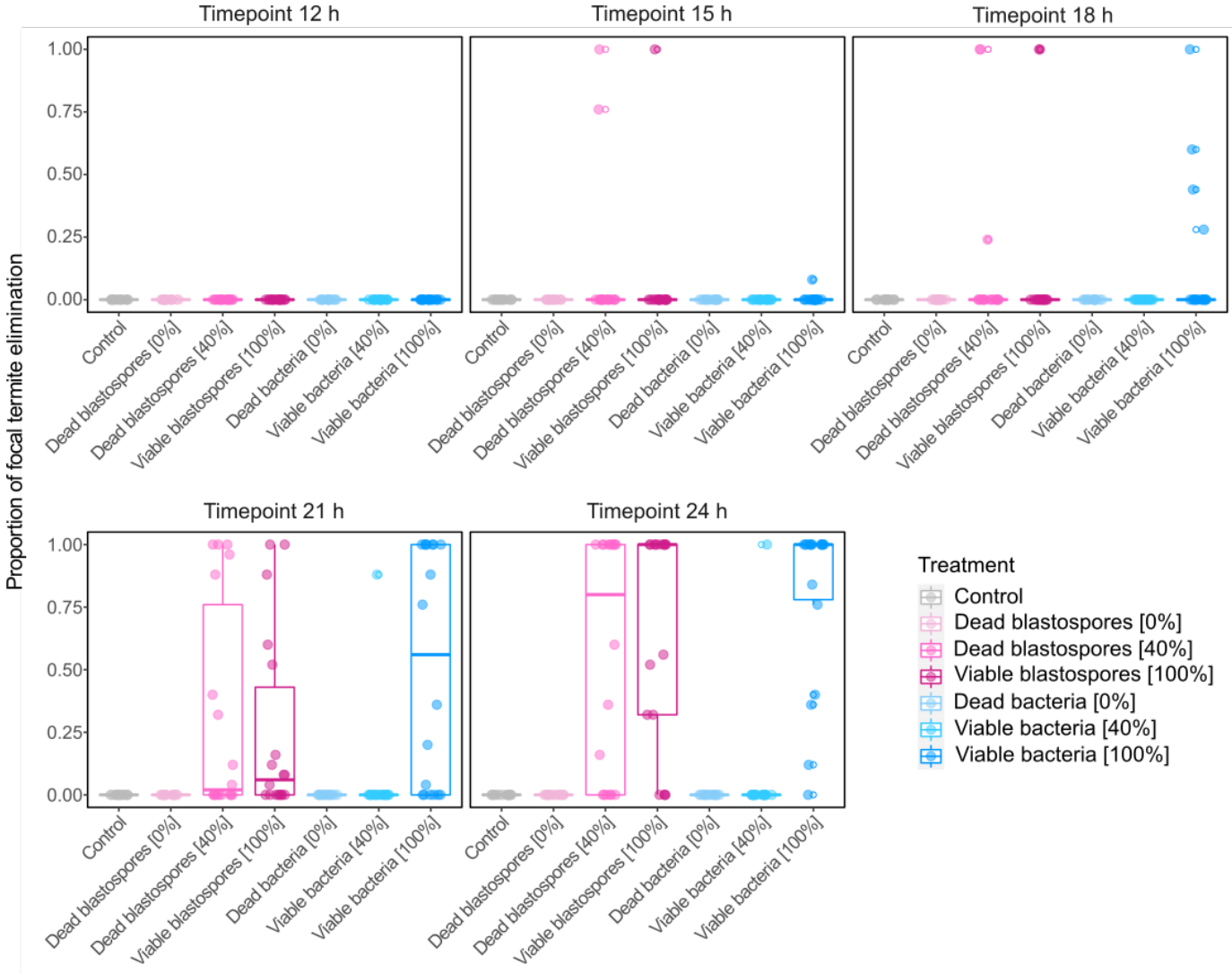
Control (Ringer)		z = 2.617 *P* = 0.186227	z = -1.871 *P* = 1.000000	z = -2.419 *P* = 0.326760	z = 1.933 *P* = 1.000000	z = 1.576 *P* = 1.000000	z = -0.002 *P* = 1.000000
0% Blastospores-			**z = -3.980** ***P* = 0.001447**	**z = -4.310** ***P* = 0.000342**	z = -0.669 *P* = 1.000000	z = -1.131 *P* = 1.000000	z = -0.003 *P* = 1.000000
40% Blastospores-				z = -0.710 *P* = 1.000000	**z = 3.603** ***P* = 0.006607**	**z = 3.333** ***P* = 0.018060**	z = -0.002 *P* = 1.000000
100% Blastospores+					**z = 3.931** ***P* = 0.001778**	**z = 3.686** ***P* = 0.004788**	z = -0.002 *P* = 1.000000
0% P. entomophila-						z = -0.438 *P* = 1.000000	z = -0.003 *P* = 1.000000
40% P. entomophila							z = -0.003 *P* = 1.000000
100% P. entomophila							

Supplementary Table S3. Z and p values from post hoc pairwise comparisons (Tukey tests using a Bonferroni correction) of the survival curves indicating the probability of being cannibalized. Statistically significant differences are indicated in bold. Empty squares mean that the comparison has already been made and can be found somewhere else in the table.

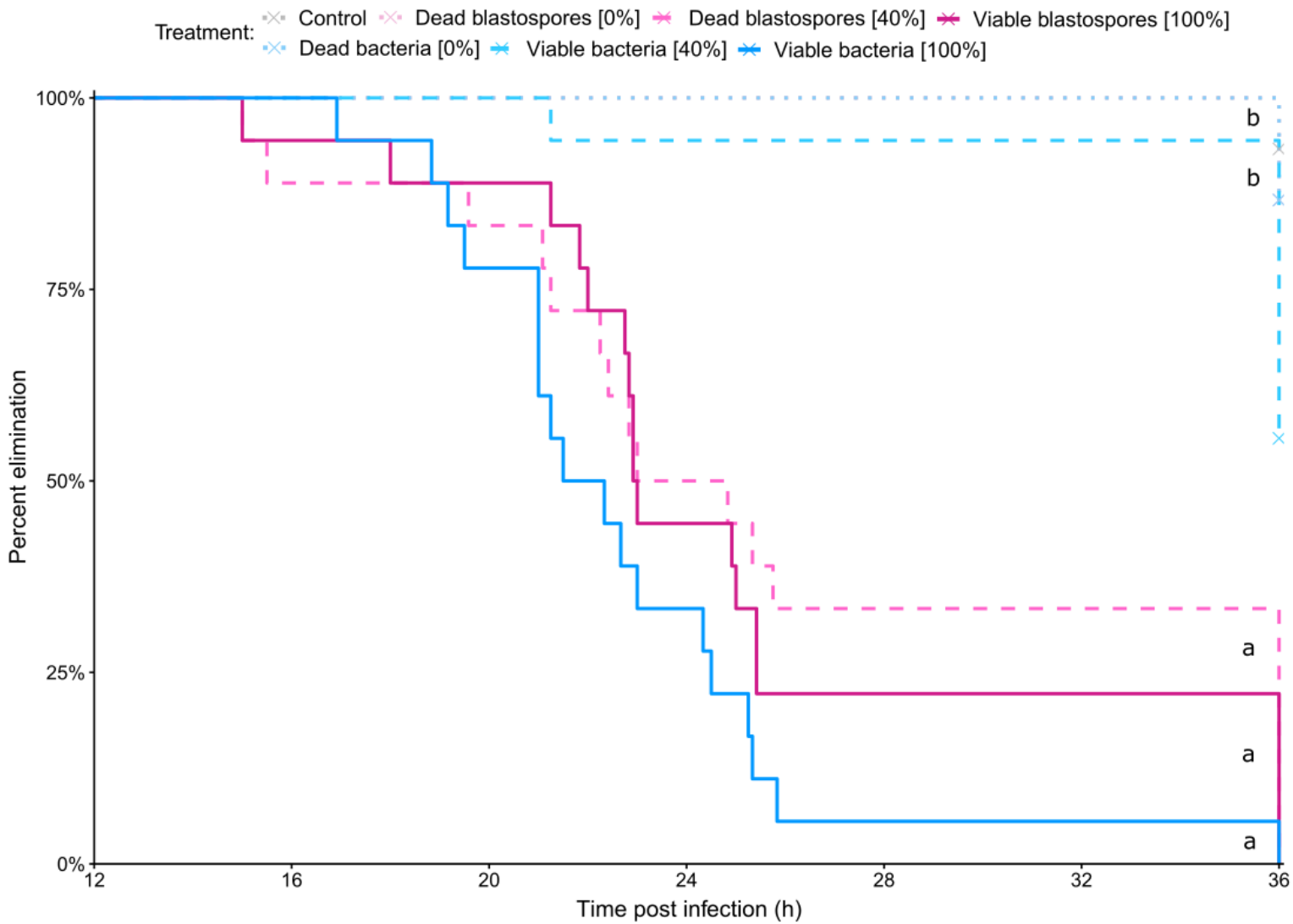
Treatment	Control (Ringer)	0% Blastospores-	40% Blastospores-	100% Blastospores+	0% P. entomophila-	40% P. entomophila	100% P. entomophila
Control (Ringer)		z = 0.000 *P* = 1.000000	**z = 4.202** ***P* = 0.000556**	**z = 4.100** ***P* = 0.000867**	z = 0.632 *P* = 1.000000	z = 1.860 *P* = 1.000000	**z = 4.621** ***P* = 8.01e-05**
0% Blastospores-			**z = 4.202** ***P* = 0.000556**	**z = 4.100** ***P* = 0.000867**	z = 0.632 *P* = 1.000000	z = 1.860 *P* = 1.000000	**z = 4.621** ***P* = 8.01e-05**
40% Blastospores-				z = -0.298 *P* = 1.000000	**z = -4.641** ***P* = 7.30e-05**	**z = -4.701** ***P* = 5.45e-05**	z = 1.260 *P* = 1.000000
100% Blastospores+					**z = -4.512** ***P* = 0.000135**	**z = -4.523** ***P* = 0.000128**	z = 1.537 *P* = 1.000000
0% P. entomophila-						z = 1.501 *P* = 1.000000	**z = 5.168** ***P* = 4.98e-06**
40% P. entomophila							**z = 5.417** ***P* = 1.27e-06**
100% P. entomophila							



Supplementary Figure S1. Cannibalism as a proportion of total visible states over time for every treatment (marked with different colors). Lower and upper hinges indicate first and third quartiles, respectively. The bold middle line represents the median. Whiskers extend to the smallest/largest value if not smaller/greater than 1.5 times the interquartile range (box length). Unfortunately, no real comparisons could be made between the values due to unresolvable problems with model convergence but the observable pattern seems to be in line with the results of the survival analysis (**Figure 3c**).



Supplementary Figure S2. Elimination as a proportion of total visible states over time for every treatment (marked with different colors). Lower and upper hinges indicate first and third quartiles, respectively. The bold middle line represents the median. Whiskers extend to the smallest/largest value if not smaller/greater than 1.5 times the interquartile range (box length).



Supplementary Figure S3. Elimination analysis of the different treatment's groups represented by different colors. The x axis indicates time post injection (in hours) while the y axis depicts percent elimination, calculated as the proportion of focal termites from every treatment group that were eliminated. Crosses

indicate the presence of right-censored data (i.e. focal termites that were not cannibalised during the experiment). A clear difference in percent elimination is visible between the controls (Ringer, dead blastospores [0%], dead bacteria [0%]) and the fungal (dead blastospores [40%], viable blastospores [100%]) and bacterial (viable bacteria [40%], viable bacteria [100%]) treatments. Treatments marked with the same letter were not significantly different, and neither "b" treatments were significantly different from the controls. The control curve is not visible due to overlap with the upper two lines (dead blastospores [0%] and dead bacteria [0%]).

Supplementary Table S4. Z and p values from post hoc pairwise comparisons (Tukey tests using a Bonferroni correction) of the percent of elimination curves indicating the number of focal termites that are not fully eliminated by the end of the experiment. Statistically significant differences are indicated in bold. Empty squares mean that the comparison has already been made and can be found somewhere else in the table.

Treatment	Control (Ringer)	0% Blastospores-	40% Blastospores-	100% Blastospores+	0% P. entomophila-	40% P. entomophila	100% P. entomophila
Control (Ringer)		z = 0.592 *P* = 1.000000	**z = 3.909** ***P* = 0.001943**	**z = 4.045** ***P* = 0.001097**	z = 0.592 *P* = 1.000000	z = 2.002 *P* = 0.950870	**z = 4.559** ***P* = 0.000108**
0% Blastospores-			**z = 4.393** ***P* = 0.000235**	**z = 4.571** ***P* = 0.000102**	z = 0.000 *P* = 1.000000	z = 1.770 *P* = 1.000000	**z = 5.249** ***P* = 3.21e-06**
40% Blastospores-				z = 0.452 *P* = 1.000000	**z = -4.393** ***P* = 0.000235**	**z = -4.388** ***P* = 0.000241**	z = 2.088 *P* = 0.773426
100% Blastospores+					**z = -4.571** ***P* = 0.000102**	**z = -4.674** ***P* = 6.20e-05**	z = 1.672 *P* = 1.000000
0% P. entomophila-						z = 1.770 *P* = 1.000000	**z = 5.249** ***P* = 3.21e-06**
40% P. entomophila							**z = 5.750** ***P* = 1.87e-07**
100% P. entomophila							

Chapter III

Inhibition of a secreted immune molecule interferes with termite social immunity

Inhibition of a secreted immune molecule interferes with termite social immunity

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Abstract

Social immune behaviors are described in a great variety of insect societies and their role in preventing emerging infectious diseases has become a major topic in insect research. The social immune system consists of multiple layers, ranging from the synthesis of external immune molecules to the coordination of individual behaviors into sophisticated collective defensive tasks. But our understanding of how complex group-level behavioral defenses are orchestrated has remained limited. We sought to address this gap in knowledge by investigating the relationship between the external activity of an important immune effector molecule in termites, Gram negative binding protein 2 (GNBP-2) and collective grooming and cannibalism. We reasoned that as an external enzyme capable of degrading entomopathogenic fungi, GNBP-2 can facilitate the spread of pathogenic molecules in the colony, and thus serve to trigger collective defenses in a manner analogous to pathogen-associated molecular signatures (PAMPs) of the individual immune system. To test whether GNBP-2 could play a role in regulating social immune behavior, we experimentally inhibited its fungicidal activity using the glycomimetic molecule, D-d-gluconolactone (GDL) and recorded collective behavioral responses to an infected nestmate. Contrary to expectations, GNBP-2 inhibition did not influence the rate or intensity of grooming of either control or fungus-infected nestmates. By contrast, we found that the probability of being harmed through defensive cannibalistic behaviors was significantly reduced by the inhibition of GNBP-2. Our findings indicate that the regulation of collective immune behaviors may depend in part on the external secretion of an enzyme originating from the individual immune system, but that other cues are also necessary.

1. Introduction

The evolutionary and ecological success of social insects can in large part be attributed to the evolution of division of labor. However, sociality also poses specific disadvantages, including increased exposure of colonies to infectious diseases (Richard, 1974; Cremer et al., 2018). The apparent disease susceptibilities associated with social live have imposed significant selection pressures on social insects to regulate the emergence and spread of disease (Schmid-Hempel, 1995; Cremer et al., 2007; Meunier, 2015). This may help to explain why epizootics that can kill entire social insect colonies are in fact quite rare (Chouvenc and Su, 2012; Schmid-Hempel, 2017). Social insect individuals are able to limit infection using their individual immune systems (Cotter and Kilner, 2010; Meunier, 2015) but they have also evolved a variety of collective disease defenses to mitigate the occurrence and dissemination of infectious diseases (Cremer et al., 2007; Wilson-Rich et al., 2009) including both behavioral and physiological adaptations (Cremer et al., 2018; Bulmer et al., 2019; Liu et al., 2019). Social actions resulting in the control or elimination of infections are examples of "social immunity." Social immunity combines defenses exhibited by the host with defenses that can be generated by surrounding relatives (Van Meyel et al., 2018). Social immunity has been termed a key property of social system evolution (Cremer et al., 2018), although a unique link between social immunity and true sociality has recently been questioned (Van Meyel et al., 2018).

Despite growing interest in the study of social immunity, we remain far from understanding how collective defensive behaviors are regulated. This is partly because social immunity represents a "distributed organ" that is comprised of a diverse array of defensive traits. For example, externally-secreted molecules derived from the individual immune system, such as toxins, acids and peptides often operate in conjunction with collective behavioral responses to protect groups against infection (Hamilton et al., 2011; Otti et al., 2014), with such molecules likely serving a critical role as a primary barrier to infection (Zasloff, 2002). In ants, termites as well as other social insect groups, behavioral defenses can be supplemented with the secretion and spread of antimicrobial substances onto body surfaces, where they function as a potent external disinfectants (Hamilton et al., 2011; López-Riquelme and Fanjul-Moles, 2013; Otti et al., 2014; He et al., 2018; Pull et al., 2018). Termites in particular can deploy a wide repertoire of social immune responses including alarm behaviors, avoidance, prophylactic, or antimicrobial secretions, burial of dead bodies, necrophagy, mutual grooming, and cannibalism (Rosengaus et al., 1998, 1999, 2011; Yanagawa and Shimizu, 2007; Chouvenc et al., 2008; Chouvenc and Su, 2010; He et al., 2018; Bulmer et al., 2019). Antimicrobial secretions in termites are produced by sternal as well as head glands, and can include antimicrobial compounds found in rectal fluids and feces (Rosengaus et al., 2011; Bulmer et al., 2019).

Termites therefore represent an excellent eusocial model for studying the evolution and function of animal immunity and sociality. However, understanding when and why different collective defenses are deployed in response to an infectious disease threat remains a

significant challenge to research. We recently showed that termites can employ a range of collective responses depending on the individual's progression along the stepwise-infection process (Davis et al., 2018). But we do not understand the underlying mechanism(s) responsible for regulating the point at which these different responses are deployed. Here, we chose to examine whether immune components synthesized and secreted by individuals could play an important role in regulating group-level behavioral responses to disease. Specifically, we focus on the role of the Gram- negative bacteria binding protein 2 (GNBP-2), which alongside the peptide Termicin, has received particular attention in termite immunity research (Lamberty et al., 2001; Yuki et al., 2008; Bulmer et al., 2009). Termicins are a class of antimicrobial peptides (AMPs) with strong antifungal activity, while GNBP-2 belongs to a class of bifunctional pattern recognition receptors (PRRs) that can recognize lipopolysaccharide (LPS) of Gram- negative bacteria and β-1,3-glucans of fungal cell walls (Bulmer et al., 2009; Hamilton and Bulmer, 2012). These proteins were first described in *Nasutitermes* (Bulmer et al., 2009) and later in *Reticulitermes* (Hamilton et al., 2011; Hamilton and Bulmer, 2012). The β-1,3-glucanase activity of termite GNBP-2 can protect termites against lethal infection by damaging conidia cell walls and thereby inhibiting germination (Rosengaus et al., 2014). GNBP-2 has been found on the insect cuticle after allogrooming as well as in nest materials, where it is likely to provide protection against generalist pathogenic fungi found in the colony environment (Bulmer et al., 2009; Hamilton et al., 2011; Hamilton and Bulmer, 2012). GNBP-2 mediated release of digested β-1,3-glucans or other fungal cell components into the nest environment could help to prevent the spread of infection by eliciting an immune response in (and thereby priming the immune defenses of) uninfected nearby termites (Bulmer et al., 2009; Hamilton and Bulmer, 2012). In addition to putative roles in termite external immunity, GNBP-2 is known to occur in the termite alimentary canal where it may act as an internal disinfectant and serve to regulate gut symbiosis during digestion (Yuki et al., 2008).

While inactivation of GNBP-2 results in suppressed immune defenses at the individual level, its involvement in collective behavior is unknown. We hypothesize that by facilitating the degradation and spread of fungal cell wall components, GNBP- 2 could act as a signal amplifier within the colony, serving to recruit nestmates to the source of infection, and therefore acting as an important molecular cue for collective defensive behavior. We test whether GNBP-2 can act as a molecular mediator of collective defense behavior by experimentally inhibiting the β-1,3-glucanase activity with D-d-gluconolactone (GDL) (Bulmer et al., 2009; Hamilton et al., 2011) and recording collective behavioral responses to nestmates infected with the fungal entomopathogen *Metarhizium anisopliae*. Entomopathogenic fungi such as *Metarhizium* have been important microorganisms in the study of insect social immunity (Rosengaus et al., 1998; Yanagawa et al., 2008; Konrad et al., 2012, 2018; Chouvenc and Su, 2012). These pathogens infect insects via cuticular penetration, leading to host death and the subsequent production of a large number of infectious spores (Vega et al., 2012; Mora et al., 2017). As facultative pathogens, they are widespread in the environment surrounding insect colonies (Cremer et al., 2018). We selected *M. anisopliae* for use in our experiments as it is a natural pathogen of termites including *Reticulitermes flavipes* (Zoberi, 1995; Dong et al., 2007) and has served as

an effective model entomopathogen in the study of virulence and termite immune defense (Chouvenc et al., 2009; Chouvenc and Su, 2010; Hamilton and Bulmer, 2012; Davis et al., 2018).

2. Materials and methods

2.1. Insect Hosts

Three *R. flavipes* colonies were used in experiments: colonies 11 + 13, 10, and X. Pieces of wood containing dense aggregations of termites belonging to these colonies were collected from the field. Colonies 11 + 13 and 10 were collected in Île d'Oléron, France, in 1999 and 1994 respectively and maintained in a dark room at 26°C, 84% humidity. Colony X was collected in 2015 in Soulac-sur-Mer, France. It was maintained in a dark room at 28°C, 83% humidity. Primary reproductives of *R. flavipes* can live to 18 years in the wild and up to 25 years in captivity (Lainé and Wright, 2003). Furthermore, secondary reproductives, which can breed amongst themselves, frequently replace primary reproductives in both native and invasive populations of this species, meaning that high levels of inbreeding are not uncommon in *R. flavipes* (Vargo and Husseneder, 2009). Colonies were kept in separate sheet metal tanks as described by Becker (1969) and had access to wood as well as sufficient damp soil to burrow. Cardboard baits were used to extract termites from their parent colonies according to Tracy (2003). After collection, we maintained termites derived from the same colony inside plastic boxes containing cellulose pads (Pall Corporation, Port Washington, United States) that had been moistened with tap water. Collected termites were kept at the same temperature as the parent colony until they were used for the experiment.

2.2. Preparation of Petri Dish Nests

The Petri dish nest was built as described elsewhere (Davis et al., 2018) to house experimental mini-colonies of *R. flavipes*. The petri dish experimental nest (94 × 16 mm) contained two cellulose pads (45.5 mm diameter, 0.9 mm thick) (Pall) which were placed on top of the two thin filter paper disc Whatman No. 5 (47 mm diameter, 0.2 mm thick). A standard microscope slide made of glass (76 × 26 mm) was then placed on top of all the filter papers. In every Petri dish, we introduced 49 healthy termites (not including the focal individual): 48 medium-to-large workers (3–5 mm body length) and one soldier. Experimental nests were sealed with parafilm to maintain a high level of humidity within petri dishes, and left in a dark room at 27°C and 70% humidity for 15 days to enable the termites to establish tunnels under the glass. To ensure a clear view into the nest a cotton swab was used to remove debris from the top surface of the glass 24 h prior to the observation experiment.

2.3. Fungal Conidia Preparation

Preparation of *M. anisopliae* conidia for use in experiments was done following Davis et al. (2018). Briefly, *M. anisopliae* DSM 1490 was maintained on potato dextrose agar (PDA) at 25°C in the dark. The conidia used in experiments were derived from a plate that had undergone a single passage from the frozen stock. Conidia from 15 days old cultures were harvested by scraping off the conidia with a sterile cotton swab moistened with sterile 0.05% Tween 80 and suspending them in sterile 0.05% Tween 80 solution. The suspension was vortexed for 30 s, then filtered through a piece of sterile miracloth (Merck KGaA, Darmstadt, D). Filtering removes hyphae and large clumps of conidia from the suspension. The filtered conidia were centrifuged for 10 min at 5,000 *g* at 4°C and the pellet was resuspended and washed three times with sterile 0.05% Tween 80, with repeated centrifugation (10 min at 5,000 *g* at 4°C) between each washing step. Conidia concentration was estimated in a BLAUBRAND Thoma counting chamber (depth 0.1 mm; BRAND, Wertheim, Germany) and the conidia suspension was adjusted to a final concentration of 1×10^8 conidia/mL and stored at 4°C until use. Conidia viability following lab culturing was evaluated by streaking with 10 µL of the same 1×10^8 conidia/mL suspension and incubating at 25°C in the dark. After 21 h of inoculation, at least 300 conidia per plate were evaluated for germination. A conidium was considered germinated if the length of the germ tube was at least half the diameter of the conidium. The germination rate was > 95% for all experiments.

2.4. Infection With Conidia or 0.05% Tween 80

We marked focal termites with Nile blue dye in order to differentiate them from colony nestmates. Nile blue dyeing was carried out following a rapid method for marking termites as described previously (Davis et al., 2018), adapted from Evans (2000). Termite workers were dipped into 2 mL microcentrifuge tubes and a sufficient quantity of 0.025% Nile blue (diluted in distilled water) was added to ensure they were completely covered. Focal termites were gently mixed for 1 min, then tipped out onto a dry Whatman No. 1 filter paper disc (90 mm diameter, 0.18 mm thick). Termites were transferred to small plastic containers, one per colony, each containing cellulose pads moistened with tap water, once they had recovered sufficiently to be able to walk. The plastic containers containing the focal termites were closed with a red tight-fitting lid to prevent desiccation and were left overnight in a dark room at 27°C and 70% humidity. Nile blue-marked termites were immersed in 1×10^8 conidia/mL suspension for 10 s and then allowed to dry onto a Whatman No. 1 filter paper disc. Infected termites were transferred individually into separate small (35 mm) Petri dishes, each containing a cellulose pad moistened with 1 mL tap water. Control termites were handled similarly but using a conidia-free solution sterile 0.05% Tween 80. The infected and control termites were incubated for 12 h at 25°C before use in the behavioral experiment. This incubation time point was chosen based on a previous study that explored termite collective behavioral responses to termites at different stages of infection (Davis et al., 2018). At 12 h post-infection, the authors recorded significantly elevated levels of allogrooming performed by nestmates, followed by a gradual transition to cannibalism, as the infected termites began

to show visible signs of sickness. The 12-h incubation time point therefore represents an optimal stage during *M. anisopliae* infection to measure the impact of treatment on two essential nestmate behaviors (i.e., allogrooming and cannibalism).

2.5. Inhibition of GNBP-2

D-d-gluconolactone (GDL) was used to block the activity of termite gram-negative binding protein (GNBP-2). GDL is a simple, non-toxic and naturally occurring derivative of glucose. It was prepared to a final working solution of 300 mM GDL and 100 mM sodium acetate (NaOAc), pH 5.0 (Bulmer et al., 2009; Hamilton et al., 2011; Hamilton and Bulmer, 2012). An equivalent control solution containing only 100 mM sodium acetate (NaOAc), pH 5.0 was prepared. GDL or control solution were applied directly in the cellulose pad food source of the nest, with which colony nestmates had direct contact.

2.6. Experimental Design

Briefly, the *R. flavipes* mini-colonies were divided into control and GDL treatments after the 15-day colony establishment period had elapsed. Twenty-four hours prior to the introduction of the focal termites into mini-colonies, the paper food source inside every petri dish nest was moistened with 900 µl of the GDL or control solution. Focal termites were comprised of either control (treated with 0.05% Tween 80) or infected (treated with 1×10^8 conidia/mL) individuals. Treatments are categorized from here on as follows: GDL+/ Ma−, GDL−/ Ma−, GDL+/Ma+, GDL−/Ma+. For each of the treatments containing *M. anisopliae* there were 15 replicates (five per colony for three colonies) and nine replicates of the control treatments (three per colony for three colonies). We recorded behavioral responses of the experimental colonies to individuals treated with a lethal dose of the entomopathogenic fungus *M. anisopliae* or a Tween 80 control solution. Infected and control termites were added individually to the Petri dish nests and then resealed with parafilm. This took ∼9 min, and the observation period began immediately after the last nest dish was sealed.

2.7. Behavioral Recording

We adopted the scan sampling method used in Davis et al. (2018). This form of instantaneous sampling allows for screening of multiple individuals (Altmann, 1974) and was used to observe the interactions between the focal termite and its nestmates. We recorded behavioral states at a single time-point during each scan of a focal termite in each experimental colony. Treatments were blinded and petri-dish locations were randomized prior to scanning. Scans typically took <1 min during which the location of the focal termite was identified and the observed behavioral state was immediately recorded. Where relevant, the number of groomers was quantified. A Samsung S7 smartphone was used as a digital voice recorder. Scans were performed every 5 min for a total of 3 h. All observations were made at 27°C, 70% humidity under bright, constant overhead light. Experimental colonies were allowed to acclimatize to light for a period of 15 h prior to introduction of focal termites. Behaviors were

classified into categories that are relevant to social immunity, and which are visually distinguishable and non-overlapping. As in Davis et al. (2018), we divided these behaviors into five different states:

Groomed by n: Focal termite is being groomed by n nestmates with no evidence of biting.

Cannibalism: Focal termite is being bitten by one or more nestmates and/or focal termite body is no longer intact.

Buried: Focal termite has had pieces of paper or feces placed on top of it. Although increasingly difficult to assess, the termite may still be alive.

Not visible: Focal termite is in a part of the nest where it cannot be observed.

Other: Focal termite is alive, intact, and unburied, but nestmates are not interacting with it. This reflects behavioral states unrelated to social immunity.

2.8. Statistical Analysis

All statistical analyses were performed using R version 3.6.0.

2.8.1. Grooming

Grooming amount(number of grooming states/total observed states) was analyzed by fitting a generalized linear mixed model using the glmer function in the R package lme4 v1.1- 21 (Bates et al., 2015), employing a binomial error structure to account for proportion data (Crawley, 2014). The model was composed of an interaction between GDL and presence of *M. anisopliae* as a fixed effect, in addition to amount of cannibalism and colony. Petri dish nest ID was used as a random effect. The anova function was used to remove fixed effect parameters that did not lead to a significant alteration in deviance, as well as to perform likelihood ratio test comparisons. The final model was tested for overdispersion in the package blmeco v1.4 using the dispersion gmer function. We carried out *post hoc* pairwise comparisons using the glht function from the multcomp package v1.4-10 with Tukey correction (Korner-Nievergelt et al., 2015a,b).

Grooming intensity was analyzed with glmer to fit a generalized linear mixed model with a Poisson error structure, using total number of groomers in each experimental nest as the response variable. As before, the model was composed of an interaction between GDL and *M. anisopliae* presence as a fixed effect, in addition to amount of cannibalism and colony. Petri dish nest ID was used as a random effect. We logged the number of grooming states, and treated these as an offset to control for the fact that each observed grooming state increased the number of groomers by at least one. As before, we used anova to compare models. Again, *post hoc* pairwise comparisons were performed using glht with Tukey correction.

2.8.2. Cannibalism

To analyze whether GDL had an impact on time spent cannibalizing (number of cannibalism states/total observed states), we fitted a zero-inflated generalized linear mixed model using the glmmTMB function in the package glmmTMB v1.0.0 (Brooks et al., 2017) employing a binomial error structure to account for proportion data. We restricted our model to GDL+/Ma+ and GDL−/Ma+, owing to insufficient data (*N* = 1 observation of cannibalism) in experimental colonies exposed to control-treated focal individuals (GDL+/Ma− and GDL−/Ma−) and subsequent model convergence issues. The conditional component of the model contained GDL as a fixed effect, in addition to amount of grooming and colony. As before, petri dish nest ID was used as a random effect. The zero-inflation component of the model contained GDL as a fixed effect. Again, we used the anova function to inspect fixed effects, as well as to perform likelihood ratio test comparisons. Although GDL did not significantly improve the model when it was included as a factor in the conditional component of the model, its inclusion did slightly improve the distribution of residuals, and so was retained in the final model.

3. Results

Following the exposure of focal termites to a control Tween 80 solution (Ma−) or *M. anisopliae* (Ma+) and isolating them for 12 h, treated termites were introduced individually to experimental nests that had been exposed to GDL or a control NaOAc solution. Behavioral patterns (**Figure 1**) in the *M. anisopliae* absent groups (GDL−/ Ma−, GDL+/Ma−) were similar regardless of GDL treatment, in that they consisted mostly of behavioral states in the “other” category (states unrelated to social immunity), with low levels of grooming, one incident of cannibalism, and no observations of burial. Behavioral patterns in the GDL−/Ma+ groups after focal termites were introduced into the experimental colonies were characterized by a concentrated phase of grooming. Cannibalism began shortly thereafter, and almost completely replaced grooming before the end of the observation period. The GDL+/Ma+ groups were also typified by initially high levels of grooming, but the intensity of grooming slowly decreased over the course of the observation period. Although cannibalism was also observed this was largely at a lower level than in the GDL−/Ma+ groups and predominantly in the final hour of the scan. We did not observe burial in any of the treatments.

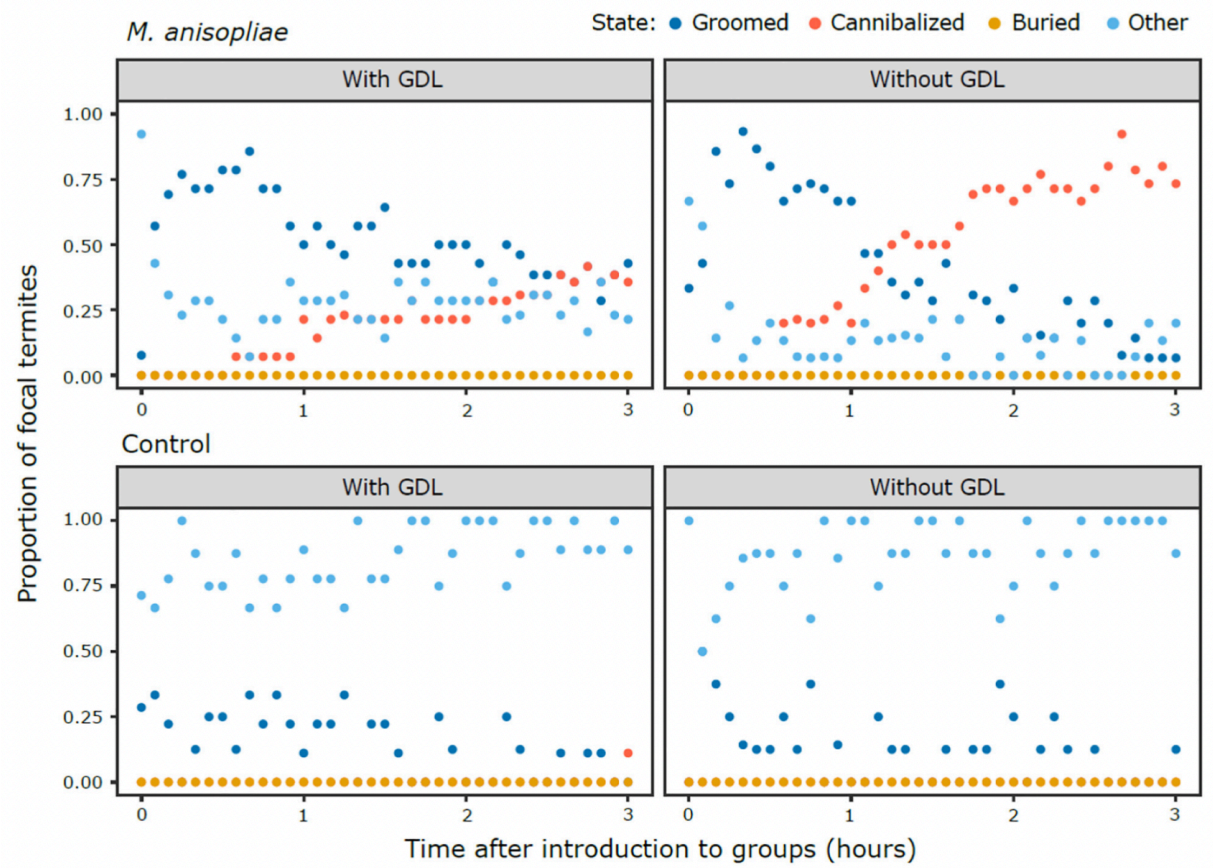

Figure 1. Patterns of behavior over the 3 h scan period, showing *M. anisopliae* (top panels) and control treatments (bottom panels) in groups of nestmates exposed to GDL (left panels) or a control solution (right panels). The proportion of focal termites that are observed in a given state at each scan (conducted every 5 min) are represented by a single point. The points overlap when more than one state was present at the same proportion across experimental colonies of a given treatment.

3.1. Grooming

The amount of grooming was significantly higher in all *M. anisopliae* treatments compared with the controls (*M. anisopliae* treatments vs. corresponding GDL−/Ma+ vs. GDL−/ Ma−, z = 7.399, P GDL−/Ma+ vs. GDL+/ Ma−, z = 6.861, P GDL+/Ma+ vs. GDL+/ Ma−, z = 7.255, P GDL+/Ma+ vs. GDL−/ Ma−, z = 7.801, $P < 0.001$) (**Figure 2** and **Supplementary Table S1**). The controls (GDL−/ Ma−, GDL+/Ma−) were not significantly different from each other and there was no significant effect of GDL treatment on proportion of grooming states. Low levels of grooming in Ma+ treatments were significantly linked with a high proportion of cannibalism states (z = -5.807, $P < 0.001$) (**Supplementary Figures S1, S2**). The negative relationship between cannibalism and grooming in Ma + treatments may explain the trend towards an increased proportion of grooming in GDL-treated experimental colonies (**Figure 2**). We also found the amount of grooming to significantly depend on colony source, with colony X displaying higher grooming amounts than either of the other two colonies (Colony X vs. 10. z = 2.902, $P < 0.011$; Colony X vs. 13+11,z=2.526,P=0.031;Colony13+11vs.10,z=0.254, P = 0.965) (**Supplementary Figure S1**).

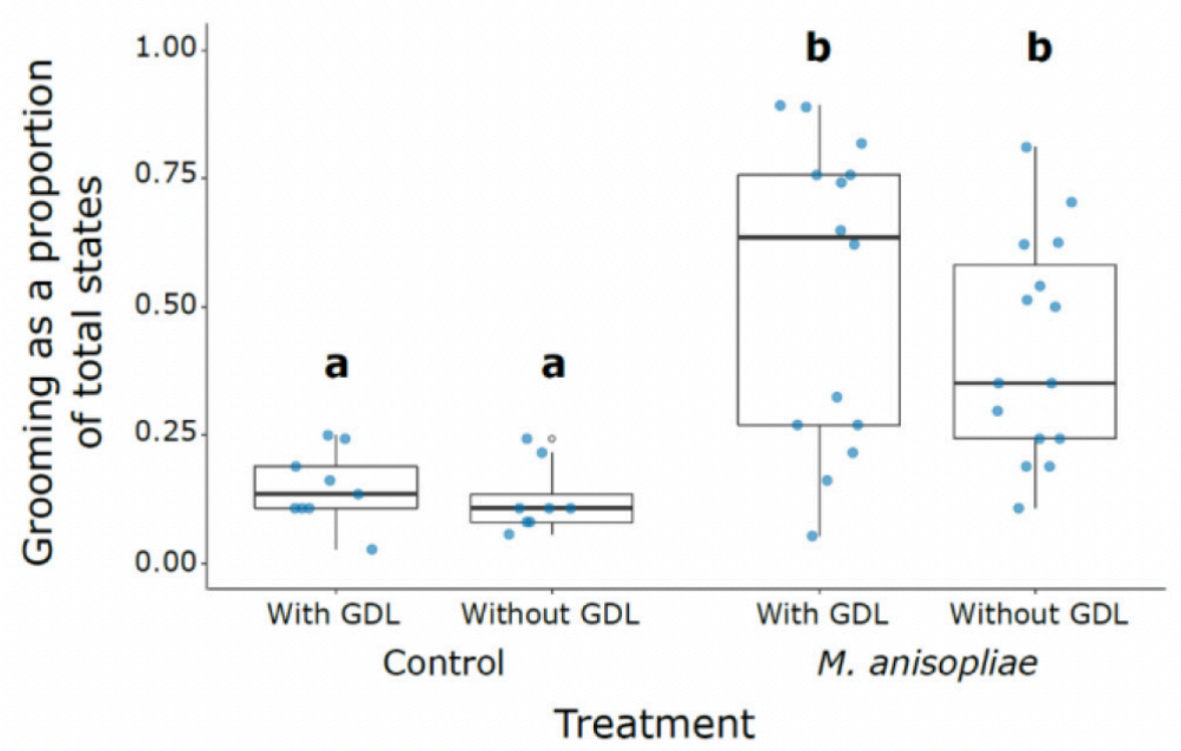

Figure 2. Grooming as a proportion of total states across treatments. Different letters indicate treatments that were significantly different following *post hoc* comparison. First and third quartiles are indicated by the lower and upper hinges. Whiskers extend to the smallest/largest value when no smaller/greater than 1.5 times the inter-quartile range from the hinge.

Intensity of grooming (number of groomers) was also significantly higher in all *M. anisopliae* treatments over the controls (*M. anisopliae* treatments vs. corresponding controls: GDL−/Ma+ vs. GDL−/Ma−. $z = 3.603$. $P < 0.002$; GDL−/Ma+ vs. GDL+/Ma−. $z = 3.213$, $P < 0.007$; GDL+/Ma+ vs. GDL+/ Ma−, $z = 3.676$, $P < 0.002$; GDL+/Ma+ vs. GDL−/ Ma−, $z = 4.015$, $P < 0.001$) (**Supplementary Figure S3** and **Supplementary Table S2**). The controls (GDL−/ Ma−, GDL+/Ma−) were not significantly different from each other and there was no significant effect of GDL treatment on number of groomers. Grooming intensity and number of groomers increased sharply in both *M. anisopliae* treatments following the introduction of focal termites, particularly in the GDL−/Ma+ treatment (**Figure 3**). Ma + groups lacking GDL also exhibited a sharper decline in both intensity and number of groomers over the course of the observation period, as grooming states were gradually replaced with cannibalism (**Figure 1**). In contrast to amount of grooming, high numbers of groomers in Ma + treatments were significantly associated with a high proportion of cannibalism states ($z = 2.651$, $P = 0.008$). Higher numbers of groomers were also observed in colony X compared with the remaining two colonies (Colony X vs. 10, $z = 2.547$, $P = 0.0291$; Colony X vs. 13+11,z=3.222,P=0.004;Colony13+11vs.10,z=-0.788, $P = 0.71024$) (**Supplementary Figures S4, S5**).

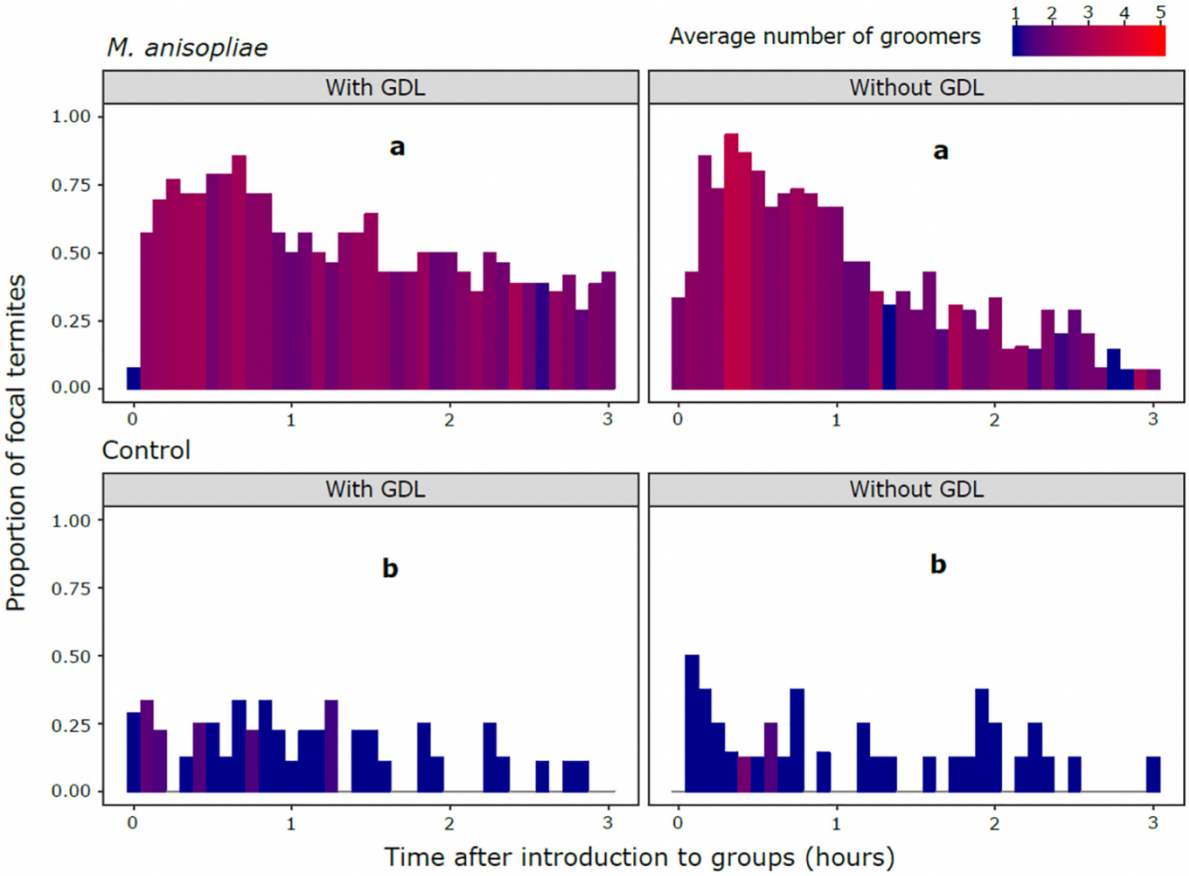

Figure 3. Proportion of focal termites observed being groomed by nestmates in each scan. *M. anisopliae* (top panels) and control treatments (bottom panels) in groups of nestmates exposed to GDL (left panels) or a control solution (right panels). Different letters indicate treatments in which overall number of groomers were significantly different following *post hoc* comparisons, after accounting for number of grooming states. Color of the fill represents the average number of groomers at each time point.

3.2. Cannibalism

The probability of being harmed during the observation period following exposure to *M. anisopliae* was significantly reduced by the inhibition of GNBP-2 (zero inflation term, GDL− vs. GDL+, z = 2.218, P = 0.027) (**Figure 4** and **Supplementary Table S3**). Furthermore, amount of cannibalism was negatively associated with amount of grooming (z = -9.053, P < 0.001). Cannibalism also varied significantly by colony, with colony 13 + 11 displaying lower amounts of cannibalism than either colonies 10 and X (Colony X vs. 10, z = -0.164, P = 0.985; Colony X vs. 13 + 11, z = 2.744, P = 0.017; Colony 13 + 11 vs. 10, z = -3.041, P = 0.007) (**Supplementary Figures S6, S7**).

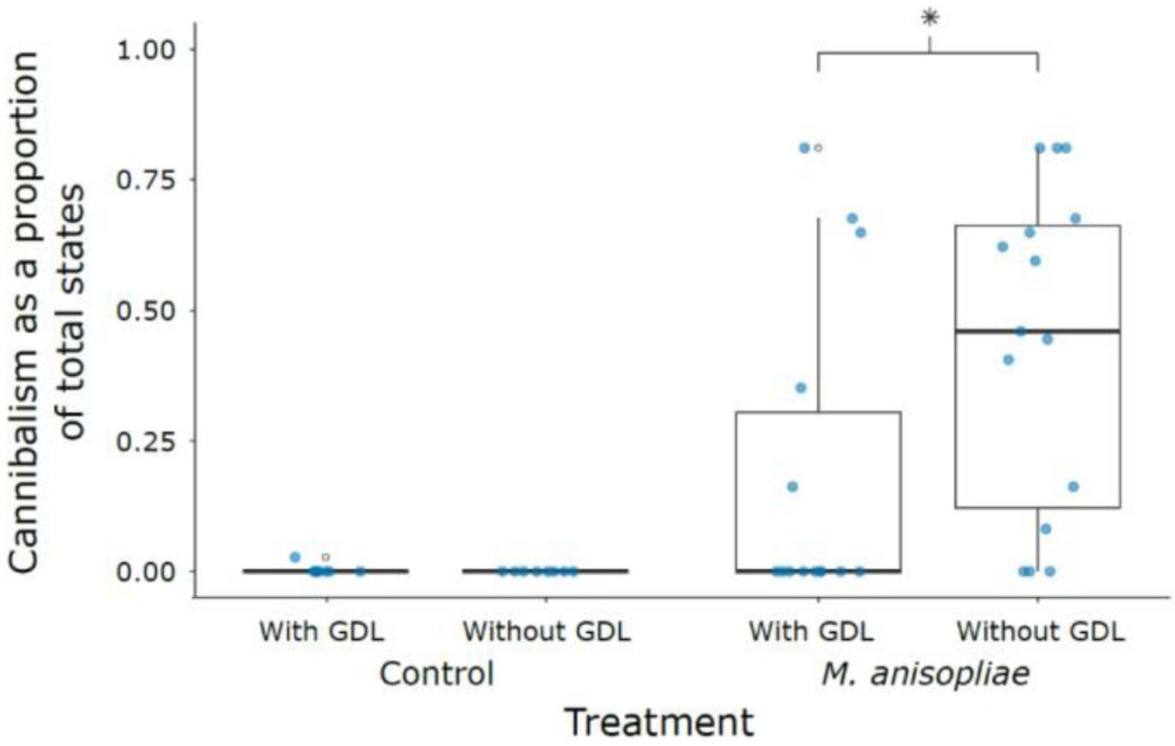

Figure 4. Cannibalism as a proportion of total states across treatments. Absence of cannibalism in *M. anisopliae* groups depends significantly on GDL treatment (indicated by an asterisk). First and third quartiles are indicated by the lower and upper hinges. Whiskers extend to the smallest/largest value when no smaller/greater than 1.5 times the inter-quartile range from the hinge.

4. Discussion

GDL treatment resulted in suppression of pathogen-induced cannibalistic behavior. But contrary to our expectations, the amount and intensity of grooming was not influenced by the application of GDL. This indicates that GNBP-2 glucanase activity can stimulate the transition from intense grooming to cannibalism but appears not to play a major role in the initial stimulation of grooming or in acting to recruit more groomers. Yanagawa et al. (2011) found that the filtrate from a suspension of *M. anisopliae* conidia was enough to induce grooming in *Coptotermes formosanus*, suggesting that grooming can be induced by the presence of fungal pathogen-associated molecular signatures (PAMPs). Interestingly, the same study did not detect any evidence of enhanced cannibalism, indicating that these are behaviors induced by signals released after infection. Davis et al. (2018) confirmed this suspicion by showing that defensive cannibalism only takes place once an infection has yielded an explicit sickness response in the termite host. In the same study, grooming was found to increase after conidia had begun to germinate, becoming even more elevated once hosts began to display signs of sickness. Similarly, in a study on ants, Pull et al. (2018) showed that pupae-derived chemical cues are used by workers to target infected pupae for destruction with poison spraying. These findings suggest that fungal PAMPs in combination with host-derived stimuli drive both grooming and destructive disinfection behaviors in social insects, as well as regulating the transition between these states. The data from the current study suggest that while GNBP-2

is unlikely to be the main mechanism by which termites detect fungal PAMPs, its activity can nonetheless influence collective behavior once the host has become sick, potentially via the release of fungal PAMPs from damaged host cuticle. It is possible that GNBP-2 inhibition does not strongly discourage grooming because termites could employ a variety of host and/or pathogen-derived signals, involving behavioral, chemical or even oscillatory cues (e.g., body vibrations) to initiate collective defense tasks (Rosengaus et al., 1999; Wilson-Rich et al., 2007; Zhukovskaya et al., 2013; Davis et al., 2018; Bulmer et al., 2019). In this scenario, although GNBP- 2 activity may itself accelerate the transition from a caring to a killing response, it represents just one component of a complex repertoire of social immune mechanisms that termites could use to regulate infectious threats exposed to the colony. Given the additional function of GNBP-2 as an internal disinfectant and putative regulator of gut symbiosis, the observed behavioral shift could represent an individual feedback response linked to disrupted digestion, rather than as a regulator of social immunity. However, it is also conceivable that GNBP-2 could fulfill both functions simultaneously.

While many studies underline the importance of collective defenses in preventing pathogen infection in termite colonies (Rosengaus et al., 1998; Traniello et al., 2002; Yanagawa and Shimizu, 2007; Zhukovskaya et al., 2013) this is to our knowledge the first to show a link between an immune molecule and collective behavioral defense. Social immune behaviors are described in several insect societies and their role in preventing emerging infectious diseases is now an established field of research (Cremer et al., 2007, 2018; Cotter and Kilner, 2010; Meunier, 2015; Kennedy et al., 2017). In addition to representing an effective model for social immunity research, our study highlights the importance of termites as a key comparative lineage to the social Hymenoptera, particularly ants, which have been a favored model for investigations into social immunity (Hughes et al., 2002; Baer et al., 2005; Ugelvig et al., 2010; Reber et al., 2011; Walker and Hughes, 2011; Pull et al., 2018). It would be particularly interesting to understand whether convergent social immune mechanisms have evolved in independent eusocial and superorganismal hymenopteran lineages. An expectation might be that externally secreted antimicrobial compounds or immune molecules can also influence collective hygienic behaviors in such groups, in addition to acting as straightforward external disinfectants.

5. Conclusion

In recent years, researchers have been trying to understand the relationships between the different layers of the social immune system: from internal physiological defenses, to the secretion of antimicrobial compounds, and culminating in the careful coordination of collective defensive behaviors. These studies are focused mainly on their evolution (Harpur and Zayed, 2013; Otti et al., 2014; Meunier, 2015; Cremer et al., 2018; Van Meyel et al., 2018) or in understanding resource allocation among the different levels of immunity to discover possible trade-offs (Armitage and Boomsma, 2010; Cotter et al., 2013; Rosengaus et al., 2013; Harpur et al., 2014; Gao and Thompson, 2015). Our aim in this study was to experimentally

test the functional relationship between these different immune layers, with the specific goal of exploring whether the "care or kill" collective defense response of a termite could be influenced by the inhibition of the fungicidal immune enzyme, GNBP-2. Although GNBP-2 represents just one piece of a larger puzzle, our findings indicate that different components of the social immune system may interact with one another. Our study describes how the orchestration of group-level hygienic behaviors could rely at least in part on relatively simple cues mediated by externally secreted molecules from the individual immune system.

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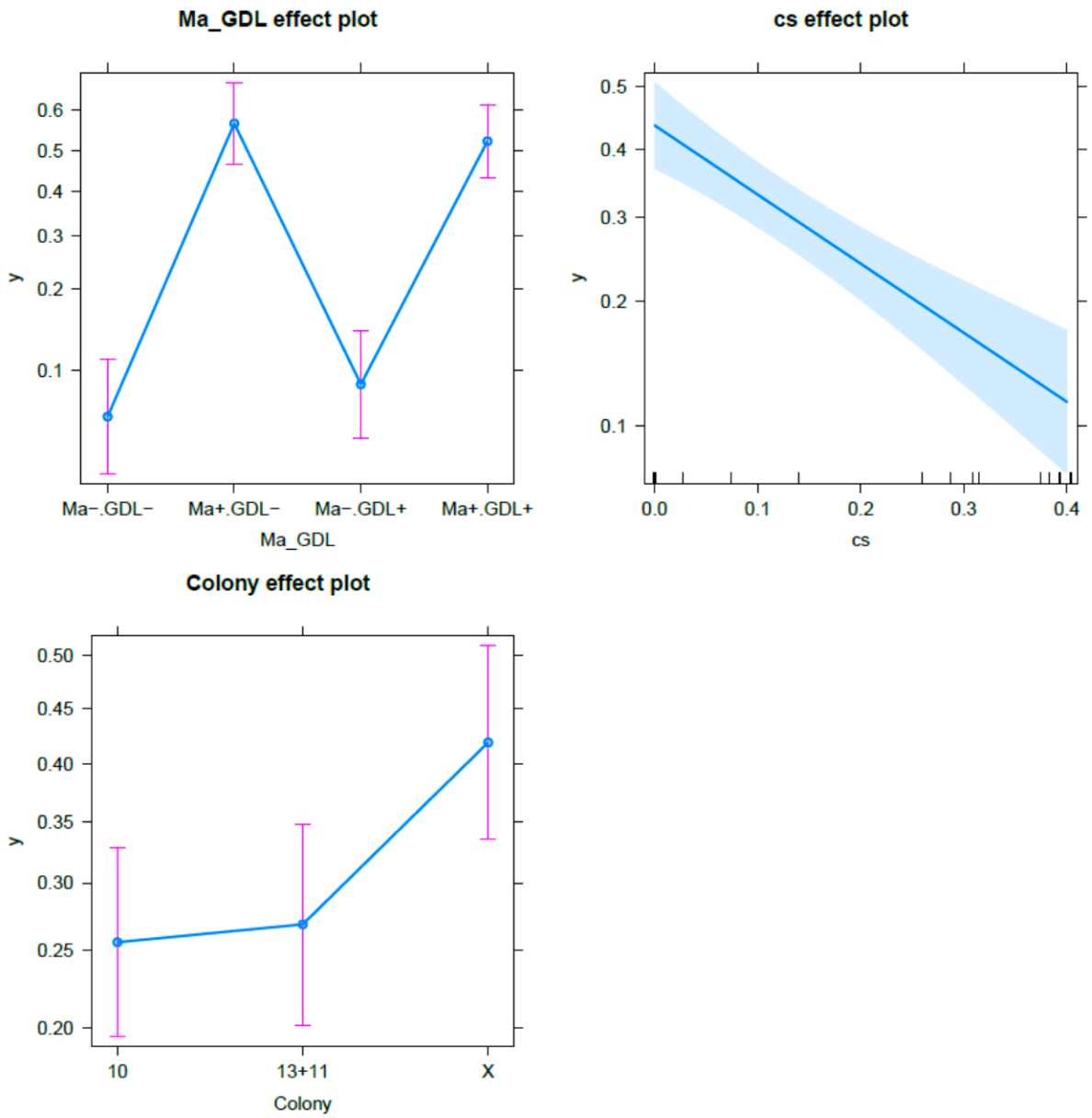
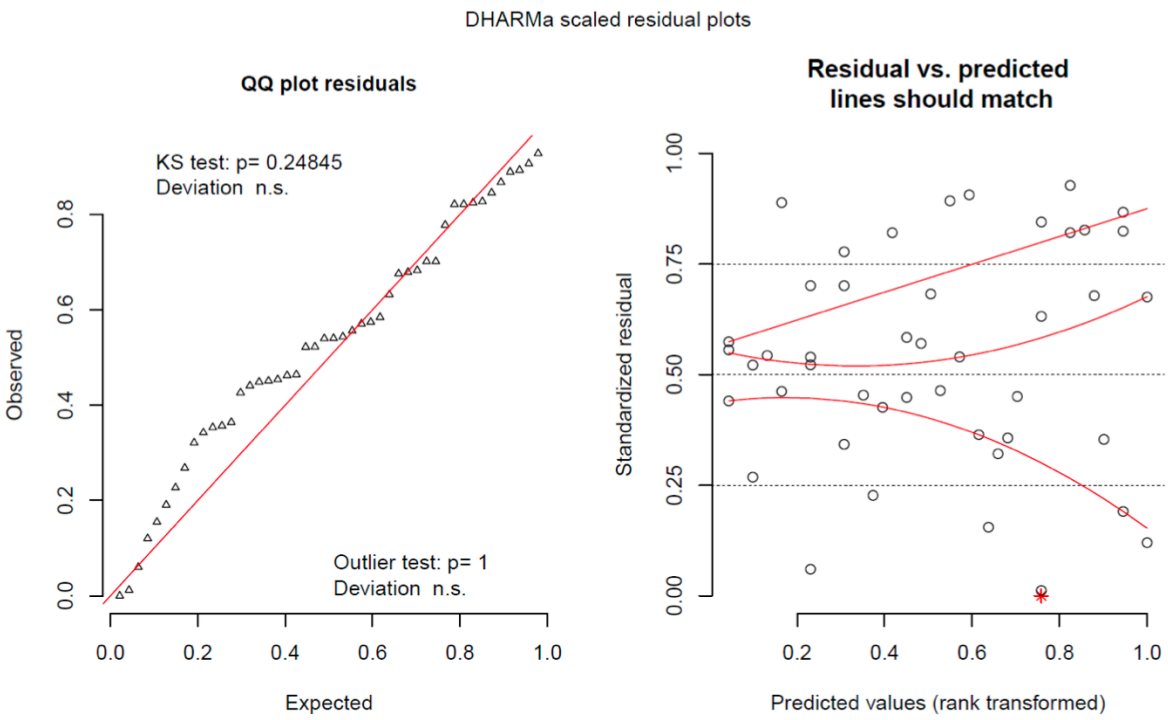
Supplementary material



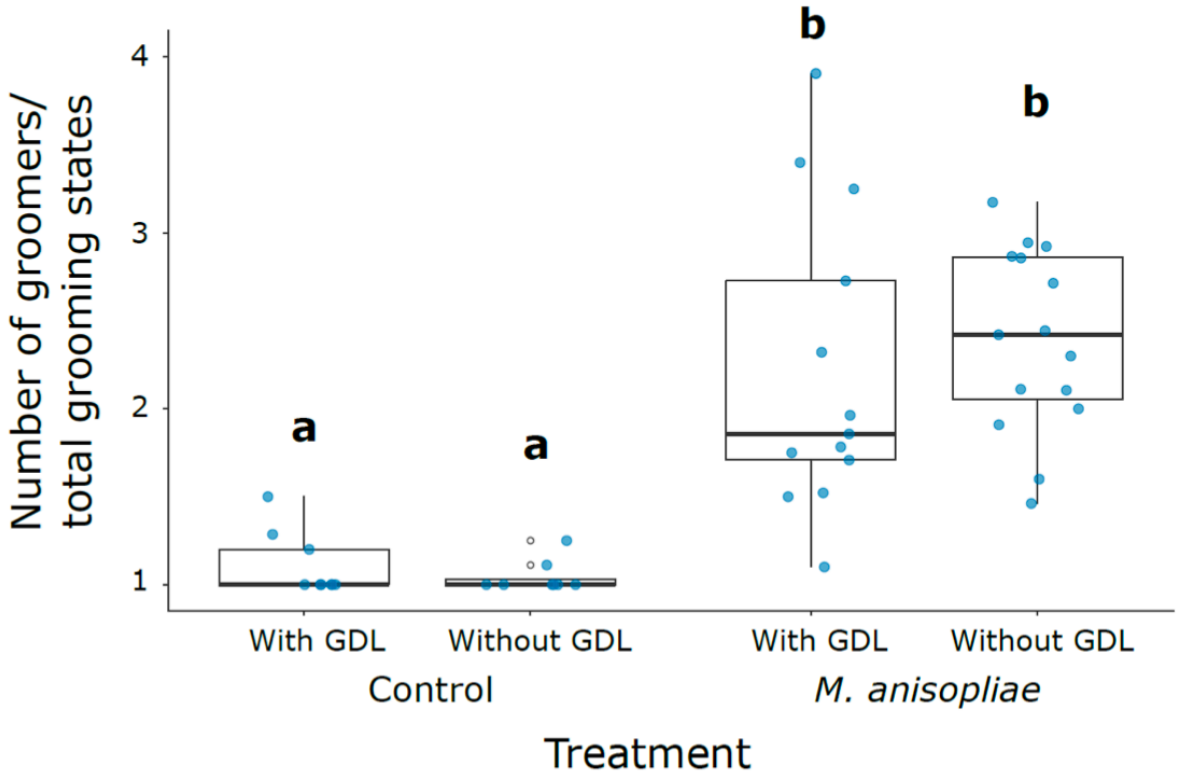
Supplementary Figure S1. Fitted effects from the generalized linear mixed model of the amount of grooming. y = number of grooming states/total observed states. *Top left*: Effects of the main interaction terms: *M. anisopliae* presence/absence (Ma+/Ma-), and GDL presence/absence (GDL+/GDL-). *Top right*: effect of cannibalism (cs) on amount of grooming. *Bottom left:* effect of colony on amount of grooming.



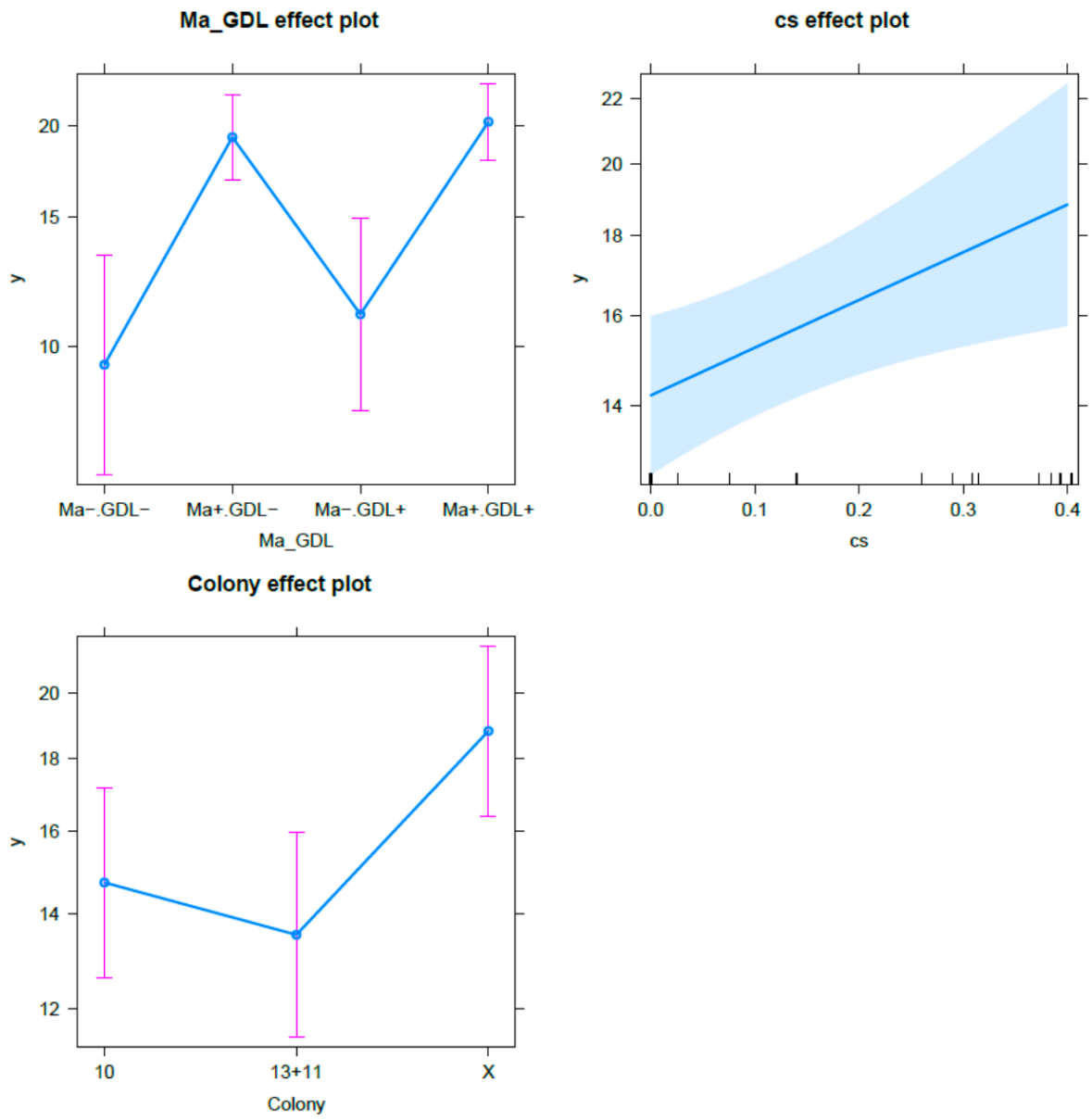
Supplementary Figure S2. Residual plots from the generalized linear mixed model of the amount of grooming. *Left:* Plot of observed versus expected residuals. *Right:* Standardized residuals versus predicted values.

Supplementary Table S1. R output from generalized linear mixed model of the amount of grooming. y = number of grooming states/total observed states.

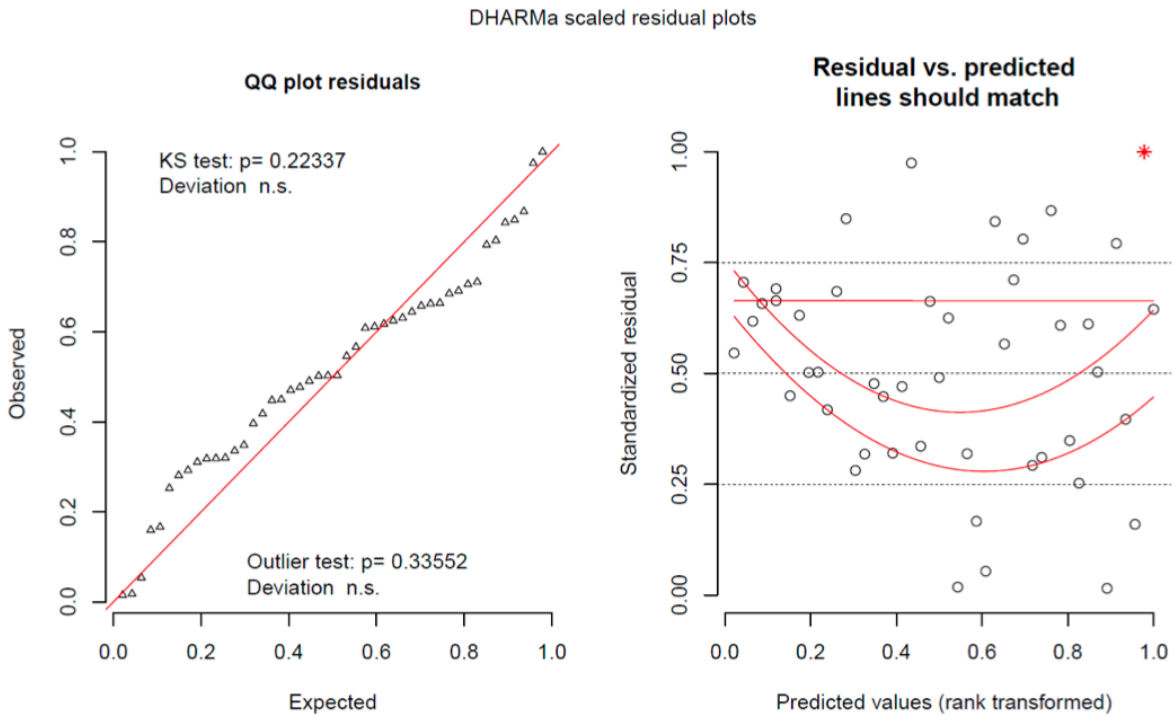
```
Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)
rMod]
 Family: binomial  ( logit )
Formula: y ~ Ma_GDL + cs + Colony + (1 | Plate)
   Data: data
Control: glmerControl(optimizer = "bobyqa")

     AIC      BIC   logLik deviance df.resid
   270.9    285.5   -127.5    254.9       38

Scaled residuals:
     Min       1Q   Median       3Q      Max
-2.22606 -0.13506  0.04143  0.28737  0.84550

Random effects:
 Groups Name        Variance Std.Dev.
 Plate  (Intercept) 0.3249   0.57
Number of obs: 46, groups:  Plate, 46

Fixed effects:
                Estimate Std. Error z value Pr(>|z|)
(Intercept)     -2.3620     0.3105  -7.607 2.81e-14 ***
Ma_GDLMa+.GDL-   2.9173     0.3847   7.583 3.37e-14 ***
Ma_GDLMa-.GDL+   0.3220     0.3762   0.856  0.39201
Ma_GDLMa+.GDL+   2.7414     0.3423   8.009 1.16e-15 ***
cs              -4.4812     0.7717  -5.807 6.35e-09 ***
Colony13+11      0.0664     0.2617   0.254  0.79970
ColonyX          0.7427     0.2559   2.902  0.00371 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) M_GDLM+.GDL- M_GDLM- M_GDLM+.GDL+ cs     C13+11
M_GDLM+.GDL- -0.630
M_GDLM-.GDL  -0.649  0.517
M_GDLM+.GDL+ -0.716  0.708        0.583
cs            0.003 -0.526        0.019  -0.257
Colony13+11  -0.348  0.014       -0.082   0.009       -0.120
ColonyX      -0.446  0.004        0.069   0.027        0.054  0.465


Ma_GDL1<-glht(model1, linfct = mcp("Ma_GDL" = "Tukey"))
summary(Ma_GDL1)

Linear Hypotheses:
                          Estimate Std. Error z value Pr(>|z|)
Ma+.GDL- - Ma-.GDL- == 0   2.9173     0.3847   7.583   <1e-04 ***
Ma-.GDL+ - Ma-.GDL- == 0   0.3220     0.3762   0.856    0.824
Ma+.GDL+ - Ma-.GDL- == 0   2.7414     0.3423   8.009   <1e-04 ***
Ma-.GDL+ - Ma+.GDL- == 0  -2.5953     0.3739  -6.940   <1e-04 ***
Ma+.GDL+ - Ma+.GDL- == 0  -0.1759     0.2806  -0.627    0.922
Ma+.GDL+ - Ma-.GDL+ == 0   2.4194     0.3293   7.347   <1e-04 ***

c1<-glht(model1, linfct = mcp("Colony" = "Tukey"))
summary(c1)

Linear Hypotheses:
                 Estimate Std. Error z value Pr(>|z|)
13+11 - 10 == 0   0.0664     0.2617   0.254   0.9651
X - 10 == 0       0.7427     0.2559   2.902   0.0102 *
X - 13+11 == 0    0.6763     0.2677   2.526   0.0309 *
```



Supplementary Figure S3. Grooming as a proportion of total states across treatments. Treatments marked with different letters were significantly different. Lower and upper hinges correspond to first and third quartiles, with whiskers extending to the smallest/largest value if no smaller/greater than 1.5 times the inter-quartile range from the hinge.



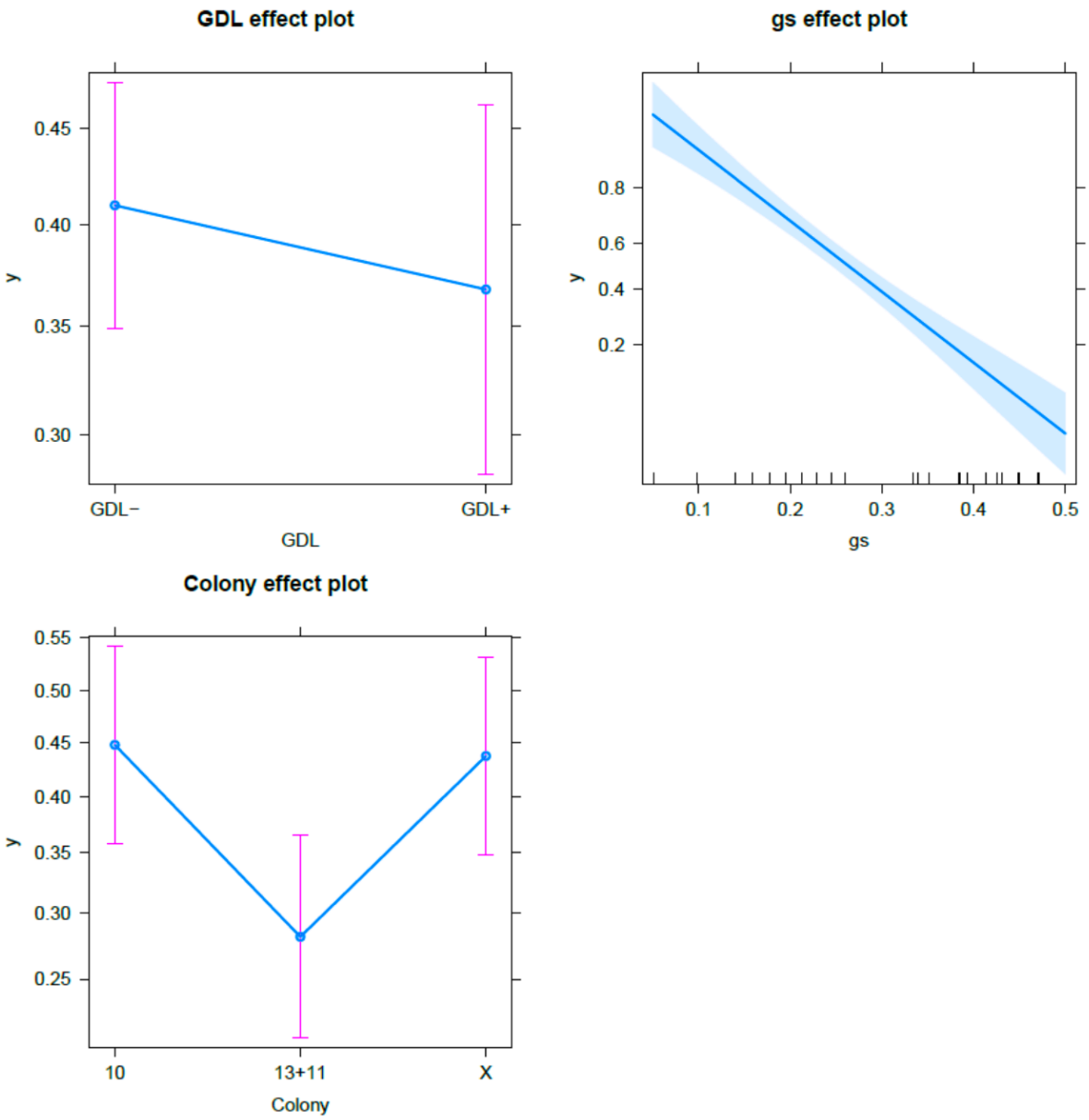
Supplementary Figure S4. Fitted effects from the generalized linear mixed model of the intensity of grooming. y = total number of groomers. *Top left*: Effects of the main interaction terms: *M. anisopliae* presence/absence (Ma+/Ma-), and GDL presence/absence (GDL+/GDL-). *Top right*: effect of cannibalism (cs) on intensity of grooming. *Bottom left:* effect of colony on intensity of grooming.



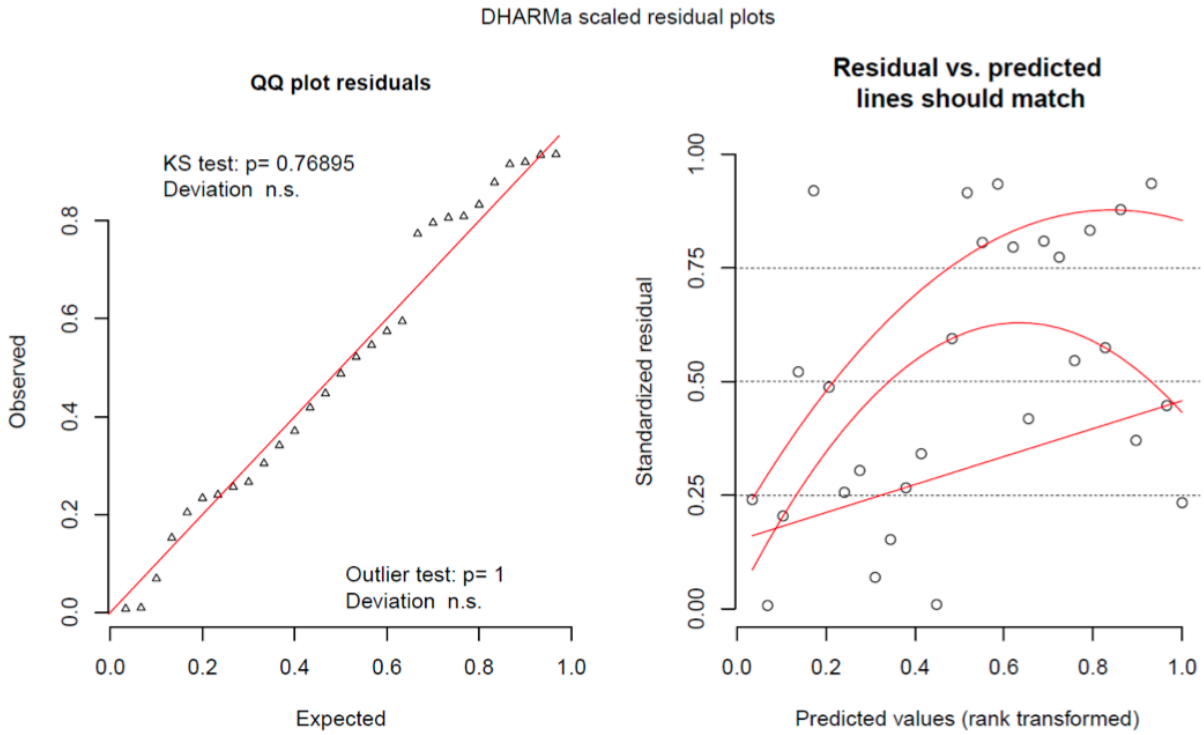
Supplementary Figure S5. Residual plots from the generalized linear mixed model of the intensity of grooming. *Left:* Plot of observed versus expected residuals. *Right:* Standardized residuals versus predicted values.

Supplementary Table S2. R output from the generalized linear mixed model of the intensity of grooming. y = total number of groomers.

```
Generalized linear mixed model fit by maximum likelihood (Laplace Approximation)
rMod]
 Family: poisson  ( log )
Formula: y ~ Ma_GDL + cs + Colony + offset(logGroomingAmount) + (1 | Plate)
   Data: data
Control: glmerControl(optimizer = "bobyqa")

     AIC      BIC   logLik deviance df.resid
   280.7    295.3   -132.3    264.7       38

Scaled residuals:
     Min       1Q   Median       3Q      Max
-1.69915 -0.35947 -0.05573  0.27644  1.74550

Random effects:
 Groups Name        Variance Std.Dev.
 Plate  (Intercept) 0.02369  0.1539
Number of obs: 46, groups:  Plate, 46

Fixed effects:
              Estimate Std. Error z value Pr(>|z|)
(Intercept)    -0.08694    0.18381  -0.473 0.636219
Ma_GDLMa+.GDL-  0.71464    0.19269   3.709 0.000208 ***
Ma_GDLMa-.GDL+  0.15846    0.23023   0.688 0.491281
Ma_GDLMa+.GDL+  0.76303    0.18459   4.134 3.57e-05 ***
cs              0.70174    0.26472   2.651 0.008029 **
Colony13+11    -0.08434    0.10709  -0.788 0.430941
ColonyX         0.24472    0.09608   2.547 0.010864 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Correlation of Fixed Effects:
            (Intr) M_GDLM+.GDL- M_GDLM- M_GDLM+.GDL+ cs     C13+11
M_GDLM+.GDL- -0.840
M_GDLM-.GDL  -0.719  0.672
M_GDLM+.GDL+ -0.874  0.880        0.703
cs           -0.032 -0.306        0.018  -0.131
Colony13+11  -0.226 -0.015       -0.040  -0.037       -0.074
ColonyX      -0.347  0.019        0.071   0.015        0.118  0.499

Ma_GDL2<-glht(model1, linfct = mcp("Ma_GDL" = "Tukey"))
summary(Ma_GDL2)

Linear Hypotheses:
                         Estimate Std. Error z value Pr(>|z|)
Ma+.GDL- - Ma-.GDL- == 0  0.71464    0.19269   3.709  < 0.001 ***
Ma-.GDL+ - Ma-.GDL- == 0  0.15846    0.23023   0.688  0.89467
Ma+.GDL+ - Ma-.GDL- == 0  0.76303    0.18459   4.134  < 0.001 ***
Ma-.GDL+ - Ma+.GDL- == 0 -0.55618    0.17465  -3.185  0.00667 **
Ma+.GDL+ - Ma+.GDL- == 0  0.04839    0.09264   0.522  0.95024
Ma+.GDL+ - Ma-.GDL+ == 0  0.60457    0.16538   3.656  0.00128 **

c2<-glht(model1, linfct = mcp("Ma_GDL" = "Tukey"))
summary(c2)

Linear Hypotheses:
               Estimate Std. Error z value Pr(>|z|)
13+11 - 10 == 0 -0.08434    0.10709  -0.788  0.71024
X - 10 == 0      0.24472    0.09608   2.547  0.02905 *
X - 13+11 == 0   0.32906    0.10214   3.222  0.00373 **
```



Supplementary Figure S6. Fitted fixed effects from the zero-inflated generalized linear mixed model of the amount of cannibalism (conditional component of the glmmTMB model). y = number of cannibalism states/total observed states. *Top left*: Effect of GDL presence/absence (GDL+/GDL-). *Top right*: effect of grooming (gs). *Bottom left:* effect of colony.



Supplementary Figure S7. Residual plots from the zero-inflated generalized linear mixed model of the amount of cannibalism. *Left:* Plot of observed versus expected residuals. *Right:* Standardized residuals versus predicted values.

Supplementary Table S3. R output from the zero-inflated generalized linear mixed model of time spent cannibalizing. y = number of cannibalism states/total observed states.

```
Family: binomial  ( logit )
Formula:          y ~ GDL + gs + Colony + (1 | Plate)
Zero inflation:     ~GDL
Data: newdata

     AIC      BIC   logLik deviance df.resid
   133.4    144.4    -58.7    117.4       21

Random effects:

Conditional model:
 Groups Name        Variance Std.Dev.
 Plate  (Intercept) 0.01324  0.1151
Number of obs: 29, groups:  Plate, 29

Conditional model:
             Estimate Std. Error z value Pr(>|z|)
(Intercept)   3.62957    0.42234   8.594  < 2e-16 ***
GDLGDL+      -0.17745    0.21546  -0.824  0.41018
gs          -12.50031    1.38081  -9.053  < 2e-16 ***
Colony13+11  -0.73008    0.24009  -3.041  0.00236 **
ColonyX      -0.04234    0.25797  -0.164  0.86964
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Zero-inflation model:
            Estimate Std. Error z value Pr(>|z|)
(Intercept)  -1.4176     0.6632  -2.138   0.0325 *
GDLGDL+       1.9401     0.8748   2.218   0.0266 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1


c3 <- glht_glmmTMB(model2, linfct = mcp(Colony = "Tukey"))
summary(c3)

Linear Hypotheses:
                Estimate Std. Error z value Pr(>|z|)
13+11 - 10 == 0 -0.73008    0.24009  -3.041  0.00666 **
X - 10 == 0     -0.04234    0.25797  -0.164  0.98525
X - 13+11 == 0   0.68775    0.25060   2.744  0.01659 *
```

Chapter IV

Blastospore based transformation of *Metarhizium anisopliae* with green fluorescence protein and resistance to glufosinate ammonium and chlorimuron ethyl

Blastospore-based transformation of *Metarhizium anisopliae* with green fluorescence protein and resistance to glufosinate ammonium and chlorimuron ethyl

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Author Contributions

JS and DPM conceived the overall idea. MAEM, TL JNT and JS designed the experiments and collected the data. MAEM and JS wrote the manuscript. All authors contributed critically to the drafts.

Unpublished manuscript.

Abstract

Entomopathogenic fungi can be considered as model systems for expression of functional genes within insects. *Metarhizium anisopliae* is an important entomopathogen widely used as a biological agent to control insect pests. Lack of an easy transformation method and selectable markers were difficulties in investigating gene functions in *M. anisopliae* by genetic engineering. Therefore, we studied whether hygromycin B (HYG), nourseothricin (NTC), geneticin (G418), fenhexamid (FEN; Teldor®), glufosinate ammonium (GFS; Basta®) and chlorimuron ethyl (CME; Classic®) prevent growth of *M. anisopliae* strains 1490 and 549 in a rapid sensitivity screening in 96-well microtiter plates. *M. anisopliae* was insensitive to HYG, NTC and G418 even at the highest concentration and to FEN concentrations up to 50 µg/mL. However, both *M. anisopliae* strains are sensitive to GFS and CME – as expected minor differences were observed between the two strains. Thus, only GFS and CME are suitable markers for transformant selection. Based on this results, inhibitory concentrations in the ranges of 100-300 µg/mL for GFS and 2.5-50 µg/mL for CME were assumed suitable for selecting transformants under different cultivation conditions. A reliable blastospore-based transformation system using GFS and CME as selective agents, *bar* and *sur* as resistance-conferring genes and a cassette for the expression of enhanced green fluorescence protein (*gfp*) was developed. The *"bar-gfp"* and *"sur-gfp"* DNA fragments were inserted into the *M. anisopliae* blastospores using the LiAc/ssDNA/PEG protocol. The following random (ectopic) integration of the DNA fragments into the genome conferred resistance to GFS and CME resulting in GFS- and CME-resistant transformants. The *bar-gfp* and *sur-gfp* expression constructs were detected by diagnostic PCR in 22 randomly chosen transformants. Both strains, MA-0001 (*baR-gfp*) and MA-0002 (*suR-gf*p), showed high levels of GFP fluorescence in conidia, hyphae and blastospores as well after colonization within the subterranean termite *Reticulitermes flavipes*. Taken together, this transformation system has proven to be functional and will serve as tool for genetic manipulation of *M. anisopliae* to discover and understand gene functions related with insect pathogenicity.

1. Introduction

Entomopathogenic fungi are important natural control agents of pest insect populations specialized in penetration through the insect cuticle, configuring an advantage if compared to other pathogens that infect the insect through ingestion (Vega et al., 2012). The genus *Metarhizium* includes the best biochemically and genetically studied entomopathogenic fungi (Fang et al., 2006; Gao et al., 2011). The genus contains, *M. anisopliae* which usually infects many insect species, including Coleoptera, Orthoptera, Lepidoptera, Diptera, Hemiptera, Isoptera and mites (Davis et al., 2018; Hänel, 1982; Roberts & St. Leger, 2004). In addition, *M. anisopliae* has been used as a model system to study fungal development, host–pathogen interactions and invertebrate immunity (Butt et al., 2016; Gottar et al., 2006; Singh et al., 2016; Syazwan et al., 2021). Commonly, the first step in the fungal infection process is the conidial adhesion to the insect cuticle due to hydrophobic interactions, followed by conidial germination and germ-tube differentiation into appressoria, which breach the cuticle by mechanical pressure and via the production of cuticle-degrading enzymes. Once inside the hemocoel, fungal cells develop yeast-like forms called hyphal bodies that invade the body cavity ultimately killing the host by one of the two strategies: (I) growing profusely in the hemolymph and physically damaging the tissues, thereby disrupting the host's physiology or (II) producing toxic metabolites that serve as immunosuppressive compounds, facilitating fungal infection. These fungal strategies have been referred to as "growth strategy" and "toxin strategy", respectively (Butt et al., 2016; Vega et al., 2012). Toxic metabolites such as destruxins were firstly isolated from *M. anisopliae*, and they are important virulence factors which accelerate the death of infected hosts (Gong et al., 2014; Kershaw et al., 1999; Rustiguel et al., 2018). After host death, hyphae emerge from within the insect cadaver to form conidiophore and produce conidia (Vega et al., 2012; Butt et al., 2016).

An efficient transformation method for *M. Anisopliae* is an indispensable tool for elucidating the function of genes involved in fungus-insect interactions, pathogenicity and delving deep into their infection strategies (Lin et al., 2011). Appropriate selection systems are important for the transformation of any foreign DNA into a fungal cell (Schmid et al., 2009). To date, genetic manipulation methods of *M. anisopliae* include the polyethylene glycol (PEG)-mediated transformation of protoplasts (Bernier et al., 1989; Fincham, 1989), and the electroporation, biolistic, and *Agrobacterium*-mediated transformation of conidia (Fang et al., 2006; Inglis et al., 2000; St. Leger et al., 1995). However, these methods can be tedious, and their transformation efficiencies are usually low. Entomopathogenic fungi often produce unicellular blastospores through yeast-like budding in certain liquid cultures, which are vegetative cells analogous to the multi-cellular hyphal bodies formed naturally in the host hemolymph (Gao et al., 2011; Vega et al., 2012). An efficient blastospore-based transformation system has been developed for introduction of foreign genes into *Beauveria bassiana*. This method readily takes advantages of the LiAc/ssDNA/PEG method (Chen et al., 2017; Ying & Feng, 2006). However, the application of this approach in *M. anisopliae*, has not

been tested so far. Development of transformation systems also allows construction of mutant strains with possible improved characteristics.

To successfully develop an efficient blastospore-based transformation system, an appropriate marker gene must be used for selection of transformants. The herbicides, glufosinate ammonium (GFS) and chlorimuron ethyl (CME), inhibit glutamine synthetase and acetolactate synthase (ALS) respectively, and thus inhibit amino acid synthesis in plants but also in microorganisms (Chaleff & Mauvais, 1984; Lin et al., 2011; Vallejo et al., 2017). The *bar* gene conferring resistance to GFS, have been isolated from *Streptomyces hygroscopicus* and the *sur* gene from the *Magnaporthe oryzae* for sulfonylurea (chlorimuron ethyl) selection were successfully used for transformation of entomopathogenic fungi, including *Paeciliomyces fumosoroseus* (Cantone & Vandenberg, 1999) and *B. bassiana* (Fang et al., 2004; Ying & Feng, 2006; Zhang et al., 2010). In this study, sought to develop a transformation system for blastospores of *M. anisopliae*. The system was assessed by integrating the genes *bar* and *sur*, conferring resistance to GFS and CME, respectively, and fused to cassettes for the expression of the green fluorescent protein (*gfp*) into the fungal genome.

2. Materials and methods

2.1. Strains and culture medium

The fungal entomopathogen *Metarhizium anisopliae* DSM 1490 was obtained from the German Collection of Microorganisms and Cell Cultures GmbH and it was used as a recipient (WT) for genetic manipulation and maintained as previously described (Davis et al., 2018). *M. anisopliae* ARSEF 549 was obtained from the Collection of Entomopathogenic fungal cultures USDA-ARS, Department of Agriculture, Agricultural Research Service, Biological Integrated Pest Management Research Unit. Strain DSM 1490 prioritizes the “toxin strategy” and ARSEF 549 the “growth strategy”. Conidia were grown on potato dextrose agar (PDA) at 25 °C in the dark until sporulation. After 15 days of incubation fresh conidia were harvested from the plate with a cotton swab moistened with sterile 0.05 % Tween 80 and suspended in sterile 0.05% Tween 80. The resulting conidia suspension was vortexed for 30 s and then filtered through a piece of sterile miracloth (Merck KgaA) to remove hyphae and large clumps of conidia. The filtered conidia suspension was centrifuged for 10 min at 5000 g at 4 °C and the pellet was resuspended and washed three times with sterile 0.05 % Tween 80. This protocol was adopted from Davis et al. (2018). The concentration was adjusted to 1 x 10^8 conidia/mL with sterile 0.05% Tween 80.

Basal synthetic medium for selection of transformants was SDNG pH 5.0 (synthetic-defined-nitrate-glucose) [0.17 % Difco™ Yeast Nitrogen Base without Amino Acids and Ammonium Sulfate (BD Biosciences), 0.3 % NaNO3, 2 % glucose, 2 % agar]. Stock solutions of selective agents were hygromycin B (HYG, Duchefa Biochemie) [41 mg/mL H2O], nourseothricin (NTC, Werner BioAgents GmbH) [100 mg/mL H2O], geneticin (G418, Sigma-Aldrich) [50 mg/mL

H2O], fenhexamid (FEN, Bayer CropScience [5 mg/mL ethanol abs.], glufosinate ammonium (GFS, ChemPur) [100 mg/mL H2O] and chlorimuron ethyl (CME, Alfa Aesar) [100 mg/mL DMF].

2.2. Transformation of *M. anisopliae*

Blastospore transformation was carried out using only the fungal strain DSM 1490. For obtaining blastospores, 1 mL of 1 x 10^8 conidia/mL suspension was inoculated in 100 mL quantities of modified liquid medium (40 g/L yeast extract, 80 g/L glucose and 0.1 % Tween 80) in 300 mL Erlenmeyer flasks in a shaking incubator for 3 days at 25 °C and 290 rpm. The suspension culture was filtered through two layers of a sterile miracloth to remove the mycelia. The filtrate was centrifuged for 5 min at 2000 g at 4 °C and the pellets containing the blastospores were washed twice with sterile ddH_2O and centrifugated at 660 g for 5 min at 4 °C. The supernatant was discarded, and the pellet resuspended in 1 mL of 0.1 M LiAc. The blastospores were precipitated one more time by centrifugation at 4,720 g for 5 min at 4 °C, and then re-suspended in 0.5 mL of 0.1 M LiAc. Blastospores were incubated at 4 °C, while concentrations were determined using a Thoma cell counting chamber and adjusted with 0.1 M LiAc to the final concentration of 1 × 10^8 blastospores/mL. Aliquots of 50 µl were supplemented in 12.5 % glycerol and stored at −70 °C for further transformations.

For transformation, we use a previously described method (Chen et al., 2017; Ying & Feng, 2006) with some modifications. The freshly prepared competent blastospores kept on ice, were harvested by centrifugation at 4720 g for 5 min and by removing the supernatant. The following agents were added in this order: 240 µL of 50 % PEG 4000, 36 µL of 1 M lithium acetate (LiAc), 10 µL of denatured genomic salmon sperm DNA (10 mg/mL), 35 µL of donor DNA (PCR amplicons, see below) or ddH2O as a control, and 35 µL of 1 M dithiothreitol. Then, the suspensions were thoroughly mixed for 1 min. The suspensions were incubated in ice for 30 min, then subjected to heat shock for 20 min at 42 °C and immediately incubated in ice for 5 min. The blastospores were harvested by centrifugation at 660 g for 5 min at 4 °C and resuspended in 600 µL of ddH_2O. Aliquots of 200 µL were spread onto SDNG agar supplemented with 250 µg/mL of GFS or with 50 µg/mL of CME resulting in two/three Petri dishes per transformation approach including controls. The Petri dishes were incubated at 25 °C in darkness until colonies appeared after approximately 5 to 8 days.

Expression constructs used in this study (**Figure 3**) were amplified in 50 µL reactions using the Q5 High-Fidelity DNA Polymerase (New England Biolabs) according to the manufacturer's instructions. The primer pair *bcniaD*-3R/Tgluc-R1 amplifies a 3.458-kb-long region from pNDP-OGG ("*bar-gfp*") and a 5.393-kb-long region from pNDS-OGG ("*sur-gfp*") (**Supplementary Figure S2**). Because of the high GC content of the bar sequence, Q5 High GC enhancer was added to this reaction according to the manufacturer's suggestions. The size and quality of the amplicons were examined by gel electrophoresis using an 1 % agarose gel and MassRuler DNA Ladder Mix (Thermo Scientific).

2.3. Genotyping of putative transformants by diagnostic PCR

Random resistant transformants were chosen for DNA extraction using the Qiagen DNA power soil kit (Qiagen, Netherlands). Two to three small cubes of mycelia were cut from the agar plate, transferred to a 1.5 mL Eppendorf to which 60 µL of C1 solution were added. This was followed by homogenizing the samples for 40 s at settings 4.0 M/S in Fast Prep-24 (MP-Biomedicals). 5 µL of Qiagen Proteinase K solution was added to the samples, vortexed and incubated at 60 °C for 2 hours. The rest of the protocol was carried out according to the manufacturer's instructions. 1 µL of DNA was checked for quality using a nanodrop 2000c (Thermo Scientific).

Diagnostic PCR was performed in standard 25 µL reactions which contained 5 µL of Red Load Taq (5x) master mix (Jena Bioscience), 0.5 µM of each primer and 1-2 ng of genomic DNA of *M. anisopliae* wild type and transformants. Thermocycling conditions according to the manufacturer's instructions were: for *baR-gfp*, 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 2 min 15 s and final elongation at 72 °C for 2 min, and for *suR-gfp*, 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, elongation at 72 °C for 2 min 12 s and final elongation at 72 °C for 2 min. The size and quality of the amplicons were examined by gel electrophoresis using 1 % agarose gels. Primer pairs for detecting the *gfp* cassette linked to baR or suR cassettes in the transformants were *bar*-hiR/T*gluc*-sR2 and *sur*-hiR/T*gluc*-sR2, respectively.

2.4. Phenotyping of transformants by fluorescence microscopy

The putative transformant colonies from SDNG plates containing 250 µg/mL of GFS and 50 µg/mL of CME were consecutively transferred individually on small SDNG plates containing GFS and CME. The expression of *gfp* gene was not only examined from mycelial mass and aerial conidia picked from the last cultures of the putative transformants but also in freshly cultured blastospores that were directly mounted onto slides and sequentially imaged in the fluorescence microscope.

To monitor mitotic stability in vivo on host insects, workers of the subterranean termite *Reticulitermes flavipes* were anaesthetized with CO_2, then injected with 41.4 nL of a solution of 5 x 10^8 blastospores/mL of either WT, GFS- or CME-resistant and *gfp*-expressing transformants directly into the hemocoel using a Nanoject II (Drummond Scientific Company, USA). The needle was inserted into the side of the thorax. Injected termites were kept individually in small Petri dishes (35 mm), each containing a Pall cellulose pad moistened with 1 mL of ddH_2O and then incubated at 25 °C until they died from mycosis and fungal outgrowth started to become visible on the insects' cuticle. Mycotized cadavers were then stored at 4 °C to limit further fungal expansion and subsequently imaged in the fluorescence microscope after 5 to 12 days.

For microscopy conidia and mycelia were suspended in Ringer solution and mounted on slides, blastospores were suspended in 0.05%-Tween 80-Solution and mounted on slides while mycotized termites were placed onto the microscope stage on a carrier without suspension. Fluorescence and light microscopy were performed with a Leica SP8 (Type TCS SP8 X - DMi8) confocal microscope. *gfp* fluorescence was examined with a gfp filter set [excitation 450-490 nm, emission 500-550 nm, dichroic mirror 495 nm], with excitation wavelength set to 490 nm at 36% intensity. Images were captured with a Leica DFC 9000 GTC camera and analysed using the Leica LAS X software (version 3.5.2.18963).

3. Results and discussion

3.1. Glufosinate ammonium (GFS) and chlorimuron ethyl (CME) as selective agents for transformant selection

Six prerequisites need to be fulfilled to successfully transform fungal cells: (1) high amounts of pure DNA, (2) highly competent cells of a suitable recipient strain that allow passage of exogenous DNA through the cell wall matrix, (3) a mechanism that delivers the transforming DNA across the plasma membrane into the cytoplasm, (4) a mechanism that safely traffics the DNA through the cytoplasm and across the nuclear envelop, (5) a mechanism that expresses the DNA integrated into the fungal genome, and (6) a recovery and selection procedure to grow and isolate positive and stable transformants (Lichius et al., 2020). The three major prerequisites that can be actively influenced by the experimenter in the laboratory are the issues (1), (2) and (6); the other issues depend on the nature of the organism.

A frequently used transformant selection method in certain fungi is based on essential nutritional genes. It requires the availability of appropriate (auxotrophic) mutants as recipient strains with a specific deficiency in the synthesis of amino acids or nucleotides or nitrate assimilation. These mutants can be maintained when grown on media containing the missing nutrient but cannot grow on minimal medium. The auxotrophic strains are transformed with DNA constructs containing the functional gene which is, for instance, fused to an expression cassette. Cells that have integrated the donor DNA into their genome will express a functional enzyme and consequently can grow on medium lacking the respective nutrient while non-transformed cells will die. There are several disadvantages to using auxotrophic markers for transformant selection because (i) a specific nutritional marker needs to be used with a specific recipient strain deficient in that nutrient, (ii) the nutritional marker may be higher/lower expressed than the normal physiological levels, and (iii) phenotypes may be altered due to the presence of the selection marker at non-physiological levels. The latter issue is particularly important when genetic studies are intended to study the fungus' metabolism or virulence. Because of these disadvantages and the lack of auxotrophic strains for *M. anisopliae*, the usage of nutritional markers had been excluded from the beginning.

Instead, the selection of transformants using dominant antibiotic/drug-resistance selection markers was considered, because any strain can be used as a recipient as far as the strain is sensitive to the compound. The prerequisites for dominant selection systems are the availability of toxic compounds and genes/alleles from any origin whose expression mediates growth in the presence of the respective compounds without side effects. For a list of commonly used dominant antibiotic/drug selection marker systems in fungi see **Supplementary Table S1** (Martín, 2015; Lichius et al., 2020). Frequently used compounds include the aminoglycosides hygromycin B (HYG), nourseothricin (NTC), and geneticin (G418) that are formed by *Streptomyces* spp. inhibiting protein synthesis in pro- and eukaryotes. The related glycopeptides phleomycin, zeocin and bleomycin are used as well but often abandoned as the rates of spontaneous mutations are too high – these compounds induce double strand breaks in the DNA and are therefore mutagenic if gene-mediated resistance (binding) is insufficient to protect the DNA. Further toxic compounds – often with a narrower action spectrum are chemically synthesized and commercially available as fungicides and herbicides. For instance, several fungicides including fenhexamid (FEN; Teldor®) target enzymes of the essential ergosterol synthesis pathway. Herbicides, including glufosinate ammonium (GFS; Basta®) and chlorimuron ethyl (CME; Classic®) block amino acid synthesis/metabolism in young plants but also in microorganisms capable of amino acid synthesis. Depending on the mode of action, selection may require an appropriate medium. Thus, a minimal medium without amino acids must be used for compounds blocking amino acid synthesis/metabolism.

	µg/mL glufosinate ammonium (GFS)												Cells/ well	µg/mL chlorimuron-ethyl (CME)										
	0	50	100	150	200	250	300	350	400	450	500	0		0	2.5	5	10	25	50	75	100	125	150	175	0 *
M. anisopliae 549	+	–	–	–	–	–	–	–	–	–	–	+	5 x 10^3	+	(+)	(+)	(+)	–	–	–	–	–	–	–	+
	+	–	–	–	–	–	–	–	–	–	–	+		+	(+)	(+)	(+)	–	–	–	–	–	–	–	+
	+	–	–	–	–	–	–	–	–	–	–	+	1 x 10^4	+	(+)	(+)	(+)	–	–	–	–	–	–	–	+
	+	–	–	–	–	–	–	–	–	–	–	+		+	(+)	(+)	(+)	–	–	–	–	–	–	–	+
	+	+	(+)	–	–	–	–	–	–	–	–	+	5 x 10^4	+	(+)	(+)	(+)	(+)	–	–	–	–	–	–	+
	+	+	(+)	–	–	–	–	–	–	–	–	+		+	(+)	(+)	(+)	(+)	–	–	–	–	–	–	+
	+	+	+	(+)	(+)	–	–	–	–	–	–	+	1 x 10^5	+	+	+	+	(+)	–	(+)	–	–	–	–	+
	+	+	+	(+)	(+)	–	–	–	–	–	–	+		+	+	+	+	(+)	–	–	–	–	–	–	+
M. anisopliae 1490	+	(+)	–	–	–	–	–	–	–	–	–	+	5 x 10^3	+	(+)	(+)	–	–	–	–	–	–	–	–	+
	+	(+)	–	–	–	–	–	–	–	–	–	+		+	(+)	(+)	–	–	–	–	–	–	–	–	+
	+	(+)	–	–	–	–	–	–	–	–	–	+	1 x 10^4	+	(+)	(+)	–	–	–	–	–	–	–	–	+
	+	(+)	–	–	–	–	–	–	–	–	–	+		+	(+)	(+)	–	–	–	–	–	–	–	–	+
	+	+	(+)	(+)	(+)	–	–	–	–	–	–	+	5 x 10^4	+	(+)	(+)	(+)	–	–	–	–	–	–	–	+
	+	+	(+)	(+)	(+)	–	–	–	–	–	–	+		+	(+)	(+)	(+)	–	–	–	–	–	–	–	+
	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+	1 x 10^5	+	+	+	(+)	(+)	–	(+)	–	–	–	–	+
	+	+	+	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	+		+	+	+	(+)	(+)	–	(+)	–	–	–	–	+

Figure 1. Rapid assay to monitor the sensitivity of two *M. anisopliae* strains to the putative selective agents glufosinate ammonium (GFS) and chlorimuron ethyl (CME).

Two wells (technical replicates) of 96-well microtiter plates per strain were filled 225 µL of blastospore suspensions (2.2 x 10^4, 4.4 x 10^4, 2.2 x 10^5 and 4.4 x 10^5 cells/mL SDNG) per well, and 25 µL of serial dilutions of stock solutions with concentrations of 100 mg GFS/mL H_2O and 100 mg CME/mL DMF resulting in the concentrations indicated the header. Growth was evaluated after 16 days of incubation at 25 °C in constant darkness: + good growth, – no growth, (+) poor growth.

To study whether the mentioned compounds prevent growth of *M. anisopliae* and are therefore suitable for transformant selection, a rapid sensitivity screening in 96-well microtiter plates was performed. For this, 3-day-grown blastospores of both *M. anisopliae* strains DSM 1490 and ARSEF 549 were harvested, washed, and resuspended in different concentrations of liquid SDNG (synthetic-defined-nitrate-glucose) medium. The medium contains glucose and nitrate as single carbon and nitrogen source, respectively, and is supplemented with vitamins, compounds supplying trace elements and salts. The antibiotic/drug compounds were added in different concentrations as indicated (**Figure 1**, **Supplementary Figure S1**). Based on observations made for related fungi, i.e. *Claviceps* and *Beauveria* spp. showing that they may tolerate high concentrations of the frequently used aminoglycosides (Haarmann et al., 2008; Lou et al., 2021; Zhang et al., 2010), we tested a large range of concentrations (0-750 µg/mL) for HYG, NTC and G418. Growth of the strains was not significantly altered even at the highest concentrations, demonstrating the insensitivity of *M. anisopliae* towards these compounds and that they cannot be used for transformant selection (**Supplementary Figure S1**). Furthermore, *M. anisopliae* strains were also found to be insensitive to FEN concentrations up to 50 µg/mL. This was already expected from the sequence analysis of the target protein ERG27 from strain ARSEF 549, which it does not contain the critical amino acid residue for binding the fungicide (not shown). The range of concentrations selected for testing the activity of the herbicides GFS and CME were based on the used inhibitory concentrations in other fungi. Our rapid assay showed that both *M. anisopliae* strains are sensitive to both compounds – as expected based on the application of these compounds for transformant selection in related species – and that the caused growth inhibitions correlated with the concentrations of the compounds and the blastospores (**Figure 1**). Minor differences were observed between the two tested *M. anisopliae* strains. Thus, strain ARSEF 549 appeared to be a bit more sensitive towards GFS and a bit less sensitive towards CME. Based on this results, inhibitory concentrations in the ranges of 100-300 µg/mL for GFS and 2.5-50 µg/mL for CME were assumed suitable for different cultivation conditions (**Figure 1**).

		µg/mL glufosinate ammonium (GFS)						Cells/ Petri dish	µg/mL chlorimuron-ethyl (CME)					
		0	100	150	200	250	300		0	2.5	5	10	25	50
M. anisopliae 549	4 days	+ | +	– | –	– | –	– | –	– | –	– | –	1.0×10^5	+ | +	+ | +	+ | +	+ | +	– | –	– | –
		+ | +	– | –	– | –	– | –	– | –	– | –	1.7×10^6	+ | +	+ | +	+ | +	+ | +	– | –	– | –
		+ | +	– | –	– | –	– | –	– | –	– | –	5.0×10^6	+ | +	+ | +	+ | +	+ | +	(+) | (+)	– | –
	8 days	+ | +	– | –	– | –	– | –	– | –	– | –	1.0×10^5	+ | +	+ | +	+ | +	+ | +	(+) | (+)	– | –
		+ | +	– | –	– | –	– | –	– | –	– | –	1.7×10^6	+ | +	+ | +	+ | +	+ | +	(+) | (+)	– | –
		+ | +	+ | +	– | –	– | –	– | –	– | –	5.0×10^6	+ | +	+ | +	+ | +	+ | +	+ | +	(+) | (+)
M. anisopliae 1490	4 days	+ | +	– | –	– | –	– | –	– | –	– | –	1.0×10^5	+ | +	+ | +	+ | +	– | –	– | –	– | –
		+ | +	– | –	– | –	– | –	– | –	– | –	1.7×10^6	+ | +	+ | +	+ | +	+ | +	– | –	– | –
		+ | +	– | –	– | –	– | –	– | –	– | –	5.0×10^6	+ | +	+ | +	+ | +	+ | +	(+) | (+)	– | –
	8 days	+ | +	– | –	– | –	– | –	– | –	– | –	1.0×10^5	+ | +	+ | +	+ | +	+ | +	– | –	– | –
		+ | +	(+) | (+)	– | –	– | –	– | –	– | –	1.7×10^6	+ | +	+ | +	+ | +	+ | +	+ | +	– | –
		+ | +	+ | +	+ | +	+ | +	(+) | (+)	– | –	5.0×10^6	+ | +	+ | +	+ | +	+ | +	+ | +	– | –

Figure 2. Growth assays of the *M. anisopliae* strains to find suitable inhibitory concentrations.
SDNG plates with different concentration of GFS and CME were prepared. Three different concentrations of blastospores (0.5 x 10^6, 0.85 x 10^7 and 2.5 x 10^7 cells/mL H_2O) were prepared and 200 µL of each one was plated. Two plates (technical replicates) per strain and conditions. Growth was evaluated after 8 days of incubation at 25 °C in constant darkness: + good growth, – no growth, (+) poor growth.

To find the optimal concentrations for the primary selection of transformants, that is spreading transformed blastospores onto selective medium, different numbers of blastospores of the two *M. anisopliae* strains were spread onto solid SDNG supplemented with 100-300 µg GFS/mL or 2.5-50 µg CME/mL (**Figure 2**). Note that the number of 5 x 10^6 blastospores per Petri dish corresponds to the selected concentration used for the transformation procedure. This test confirmed the lower sensitivity of the strain DSM 1490 towards GFS compared to strain ARSEF 549. As the growth of both strains was completely inhibited on SDNG containing 250 µg GFS/mL and 50 µg CME/mL, these concentrations were used for screening for resistant colonies in the following transformation approaches.

3.2. Ectopic integration of *gfp* expression constructs in *M. anisopliae* 1490 via blastospore-mediated transformation.

The compounds GFS and CME are used as herbicides as they inhibit amino acid synthesis in plants, but they also can prevent growth of microorganisms including *M. anisopliae*. The genes *bar* and *sur* conferring resistance to GFS and CME i.e. their expression allows transformed naturally sensitive cells to grow, originate from the bacterium *Streptomyces hygroscopicus* and the fungus *Magnaporthe oryzae*, respectively. The *bar* product encodes a phosphinothricin N-acetyltransferase that detoxicates the chemical compound GFS and the *S. hygroscopicus*-derived toxic compound phosphinothricin tripeptide (bialaphos) by acetylation of the shared amino group. GFS and bialaphos inhibit the glutamine synthetase resulting in accumulation of toxic levels of ammonium and the disruption of amino acid synthesis. The *sur* product is an insensitive allele of the drug target i.e. it carries a target-site mutation in the acetolactate synthase (ALS) which is essential for the synthesis of branched-chain amino acids in bacteria, fungi and plants. *Sur* replaces the essential sensitive allele of the strain during cultivation with CME (Sweigard et al., 1997). Therefore, two selection marker systems i.e. phosphinothricin and chlorimuron ethyl resistance will allow for introducing double mutations in *M. anisopliae*.

Modular cloning vectors of the pNDR-XXX series (Schumacher, 2012) containing *bar* and *sur* for selection with GFS and CME were recently cloned and tested for functionality in the rock-inhabiting fungus *Knufia petricola* (Erdmann et al., 2022a). Briefly, *bar* and *sur* were amplified from pCB1524 and pCB1532 (McCluskey et al., 2010; Sweigard et al., 1997) with primers generating overlaps with P*trpC* or T*niaD* and the obtained amplicons were assembled in *Saccharomyces cerevisiae* with the pND-OGG backbone yielding pNDP-OGG (baR) and pNDS-OGG (suR) (**Supplementary Figure S2**). Here, the resistance genes are flanked by the same

regulatory sequences that are *Aspergillus nidulans* P*trpC* and *Botrytis cinerea* T*niaD*. Both plasmids contain an identical *gfp* expression cassette (P*oliC*::*gfp*::T*gluc*) consisting of *gfp* [optimized variant for *B. cinerea*; (Leroch et al., 2011)] and *A. nidulans* P*oliC* and *B. cinerea* T*gluc* as regulatory elements known to mediate high constitutive gene expression in many fungi. The modular structure of the plasmids of the pNDR-XXX series enabled the amplification of the *gfp* cassettes fused to either the baR cassette (“*baR-gfp”*) or the suR cassette (“*suR-gfp”*) by using the same primer pair (*bcniaD*-3R/T*gluc*-sR1) and pNDP-OGG or pNDS-OGG as template (**Figure 3**). Note that the amplification of the GC-rich *bar* required modification of the PCR protocol e.g. the addition of DMSO or DNA polymerase-specific ‘GC enhancer’ mixtures.

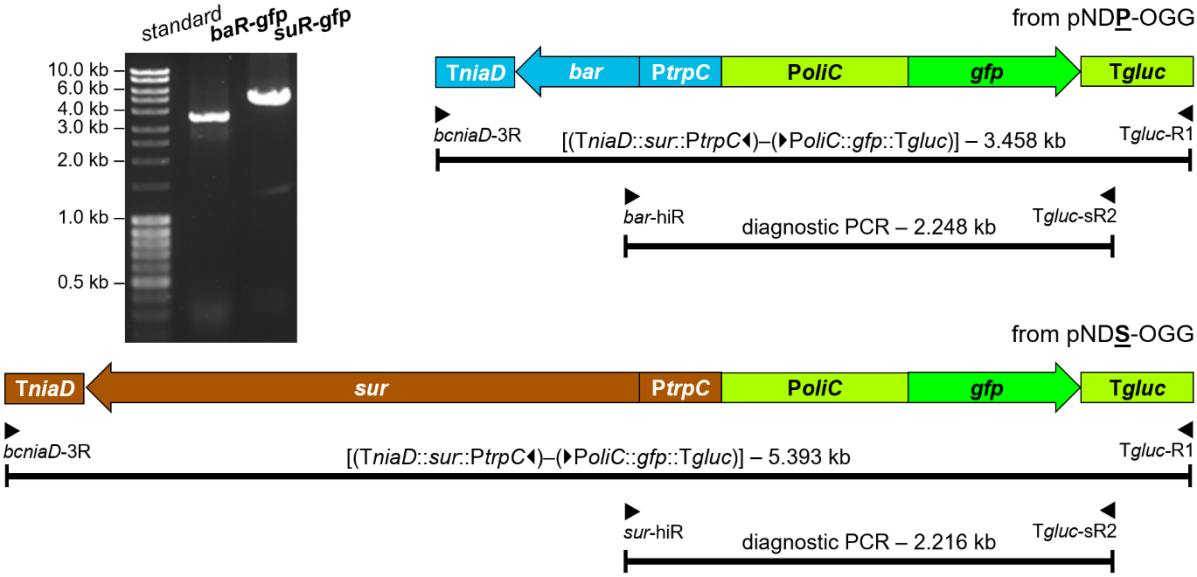

Figure 3. Expression constructs for ectopic integration into the *M. anisopliae* genome.
The constructs consist of the identical *gfp* expression cassette (P*oliC*::*gfp*::T*gluc*) and structurally identical resistance cassettes (P*trpC*::*Rgene*::T*niaD*) for mediating growth in presence of toxic concentrations of glufosinate ammonium via expression of *bar* (encodes a phosphinothricin N-acetyltransferase) or chlorimuron ethyl via expression of *sur* (encodes a sulfonylurea-insensitive acetolactate synthase). The constructs – in the following briefly called *bar-gfp* and *sur-gfp* – were amplified by PCR using the primer pair *bcniaD-3R/*T*gluc-sR1* and the plasmids pNDP-OGG and pNDS-OGG [(Erdmann et al., 2022b); see Supplementary Figure S2], respectively, as template. Aliquots (3 µL) of the amplified constructs were separated in an 1% agarose gel for quality control (top left). Primers used for genotyping of putative transformants by diagnostic PCR are indicated. Promoters P*oliC* and P*trpC* originate from *Aspergillus nidulans*, terminators T*gluc* and T*niaD* from *Botrytis cinerea*, and resistance genes bar and sur derive from *Streptomyces hygroscopicus* and *Magnaporthe oryzae*, respectively.

Aiming at an easy and fast method to transform *M. anisopliae*, we focussed on the implementation of a protocol using blastospores – as it was done in *B. bassiana* (Chen et al., 2017; Ying & Feng, 2006) – rather than protocols enclosing the generation and recovery of sensitive protoplasts or using *Agrobacterium tumefaciens* as vector. The blastospore-based transformation method is a variant of the LiAc/ssDNA/PEG protocol used for transformation

of the budding yeast *Saccharomyces cerevisiae* (Schiestl & Gietz, 1989) which uses a combination of lithium acetate (LiAc), single-stranded (ss) carrier DNA e.g. denatured salmon sperm DNA, and polyethylene glycol (PEG). Based on the procedures reported for *B. bassiana* and *S. cerevisiae,* and the growth characteristics of *M. anisopliae*, we developed the protocol described in materials and methods section. Briefly, the procedure involves four major steps: (1) cultivation of the recipient strain starting from conidia to obtain blastospores, (2) preparation of competent blastospores by treatment with LiAc, (3) transformation with linear DNA comprising the resistance cassette by treatment with ssDNA and PEG, and (4) subsequent selection of resistant colonies as putative transformants using SDNG agar supplemented with the respective selective agent.

Blastospores of *M. anisopliae* 1490 were transformed with the amplified *bar-gfp* and *sur-gfp* constructs in two independent experiments, both resulting in resistant colonies when DNA was added. No growth was observed for the negative controls, which were blastospores "transformed" with H_2O instead of DNA. The GFS-resistant (*baR)* and CME-resistant (*suR*) colonies became visible five days after transformation and grew well after eight days (**Figure 4A**). Examples of the transformation and control plates are shown. In total, thirty-eight resistant colonies for *baR-gfp* (MA-0001) and thirty-nine for *suR-gf*p (MA-0002) were obtained (**Figure 4B**). Mycelia of these colonies were transferred to SDNG medium containing the respective selective agent to confirm the resistance phenotype (not shown). Furthermore, in total eleven *bar-gfp* and eleven *sur-gfp* (putative) transformants from both transformations were randomly selected for genotyping by diagnostic PCR. The genomic DNA of these putative transformants were extracted, checked for quality and quantity in an agarose gel (not shown) and used as template for a PCR using the primer pairs *bar*-hiR/T*gluc*-sR2 and *sur*-hiR/T*gluc*-sR2, respectively (**Figure 3**). For control, a PCR with the primer pair NS7/LR5 amplifying a 1.809-kb-long region of the conserved rDNA operon (**Figure S3**) was run in parallel on all DNA samples including that of the non-transformed recipient strain 1490 (**Figure 4C**). The expected amplicons with sizes of 2.248 kb (*bar-gfp*) and 2.216 kb (*sur-gfp*) were obtained for all analyzed resistant transformants demonstrating that the DNA (expression constructs) was taken up and stably (and ectopically) integrated into the genome of *M. anisopliae* to confer resistance towards GFS and CME, respectively.

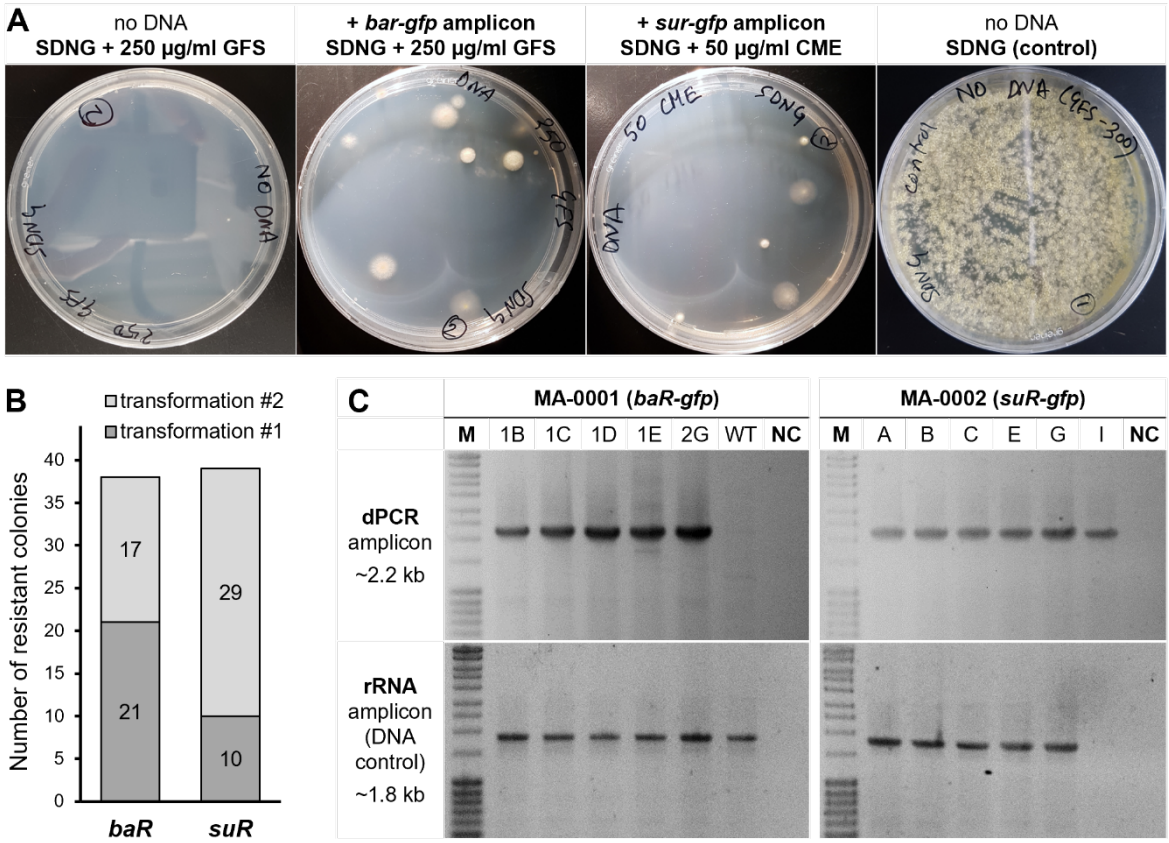

Figure 4. Transformation of blastospores of *M. anisopliae* (1490) with *baR* and *suR* expression constructs.

A: Resistant colonies derived from blastospores transformed with *baR-gfp* or *suR-gfp* on solid SDNG medium containing 250 µg GFS/mL or 50 µg CME/mL after eight days of incubation at 25 °C in darkness. Controls were competent blastospores "transformed" with H_2O instead of DNA and spread onto selective (on the left) and non-selective SDNG medium (on the right) of glufosinate ammonium and chlorimuron ethyl cultured with blastospores containing bar-gfp and sur-gfp amplicon and control (no resistant marker) as well. **B:** Number of resistant colonies obtained from two transformations of *M. anisopliae* 1490 blastospores with *bar-gfp* and *sur-gfp* amplicons. **C:** Diagnostic PCR confirms the integration of *baR-gfp* and *suR-gfp* expression constructs into the genome of *M. anisopliae* 1490. Expected amplicons for MA-0001 (*baR-gfp*) and MA-0002 (*suR-gfp*) were 2.248 kb and 2.216 kb respectively (**Figure 3**). dPCR – diagnostic PCR, rRNA – DNA control PCR, NC – negative control (no template).

3.3. Resistant transformants express *gfp* in conidia, hyphae, blastospores and during colonization of the host.

All GFS- and CME-resistant (MA-0001 and MA-0002) and *gfp*-expressing transformants obtained displayed uniformly high levels of green fluorescence in conidia, hyphae and blastospores. As expected, no GFP fluorescence was detected the *M. anisopliae* wild-type (WT) cells (**Figure 5C, Supplementary Figure S4B, D**). No significant differences in morphology between the WT, MA-0001 and MA-0002 was observed (**Figure 5A**). After injection of blastospores within the termite hemocoel, a successful infection was initiated leading to host death and subsequent outgrowths from termite cadavers. Fungal outgrowths displayed a

strong expression of green fluorescence in MA-0001 and MA-0002-injected termites but not in the WT-injected termites (**Figure 5D**). Thus, the transformants produced the green fluorescence protein *in vitro* and *in vivo*.

Introduction of the *gfp* gene as visual markers has been widely used in transformation systems of entomopathogenic fungi, conferring several advantages for studying the obtained transformants. First, expression of *gfp* serves as a handy, visual confirmation method of a successful transformation (Ying & Feng, 2006; Cao et al., 2007; Zhang et al., 2010; Padilla-Guerreiro & Bidochka, 2017). The use of *gfp*-labelled transformants can then be used to assess infections of insect hosts which give the possibility to visualise different infection stages within the host body, as already done with other species of the *Metarhizium* genus, like *M. brunneum* or *M. robertsii* (Alkhaibari et al., 2018; Reingold et al., 2021). Expression of *gfp* gene using *B. bassiana* was detected in mealworm and peach aphid infected cadavers (Jin et al., 2010; Kim et al., 2013). Second, *gfp*-expression has shown to represent a practical tool for delineating with spatial and temporal traits of gene activity for specific genes involved in pathogenicity (Jin et al., 2010; Wang et al., 2021) and for bioassaying characteristics like virulence against different hosts (Inglis et al., 2020).

As we see, the possible applications of a transformation system with a *gfp*-marker for *M. anisopliae* are manifold. These approaches can be used on one side to express other functional genes, such as antimicrobial peptides (AMP), edible vaccines, and so on. On the other hand, transformation of *M. anisopliae* also provides a powerful tool for elucidating the function of pathogenicity genes, including targeted disruption of toxic metabolites like destruxins. Generally, *gfp*-labelled *M. anisopliae* strains are likely to facilitate future epidemiological and host colonization (within-host infection process) studies in termites and it will also help us to distinguish how different infection strategies from the pathogen affect host responses.

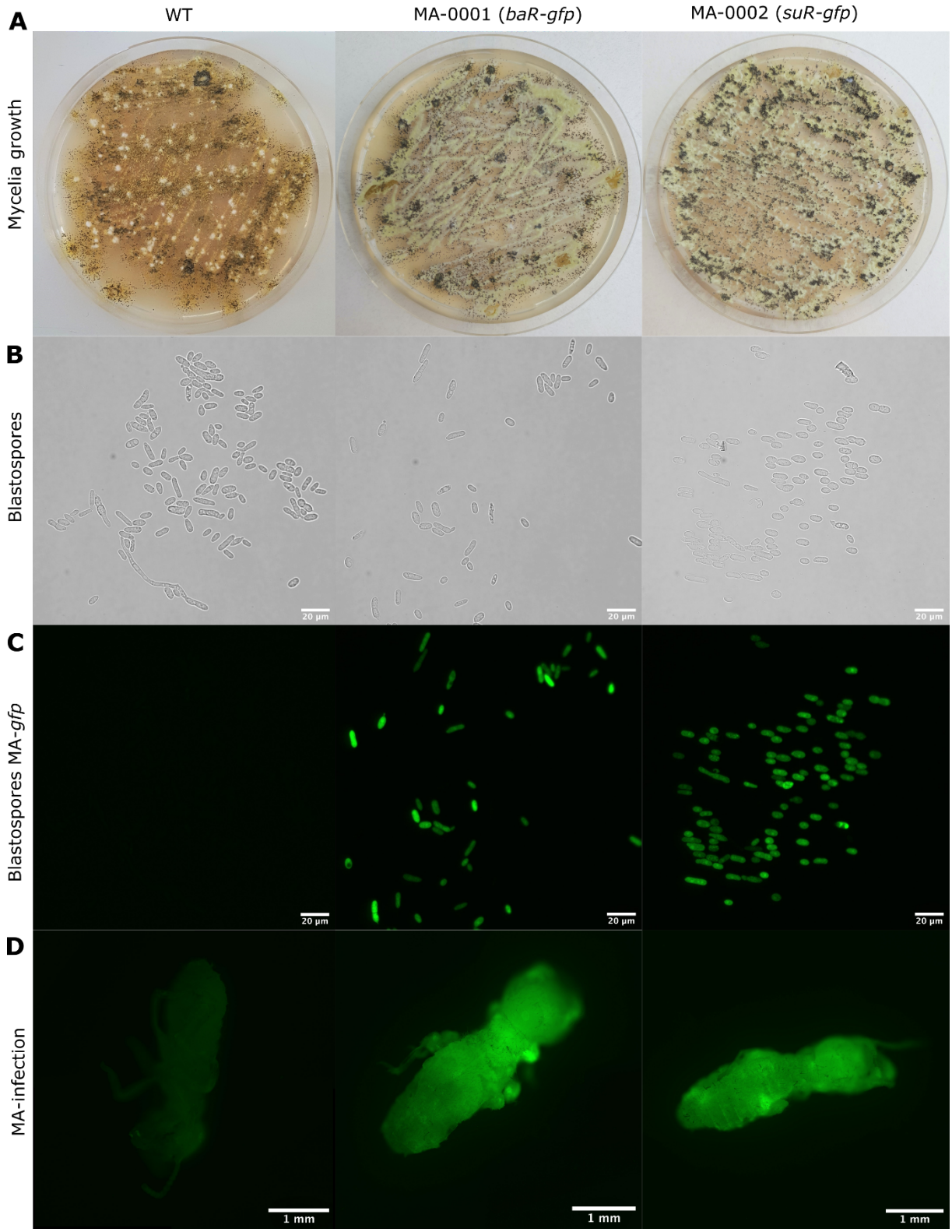

Figure 5. Verification of the expression of enhanced green fluorescence protein (*gfp*) gene in *M. anisopliae* 1490.

A: Mycelial growth of wild type (WT) and *gfp* transformants on solid SDNG medium containing 250 µg GFS/mL or 50 µg CME/mL after incubation for eight days at 25 °C in constant darkness. **B:** Brightfield images of *M. anisopliae* blastospores from WT and *gfp* transformants (3 days of culture in liquid media) under a fluorescence microscope. **C:** Detection of GFP fluorescence in WT and *gfp* putative transformants (3 days of culture in liquid media) under a fluorescence microscope. Scale bars=

63 x. **D:** WT and MA-*gfp*-infected termite cadavers 4 days after injection of blastospores. At this stage, fungal outgrowth existing on top of the cadaver after successful infection of the host was checked for GFP fluorescence. *Gfp* ectopically integrated into the genome of *M. anisopliae* was stably expressed within the host. Scale bars= 5 x.

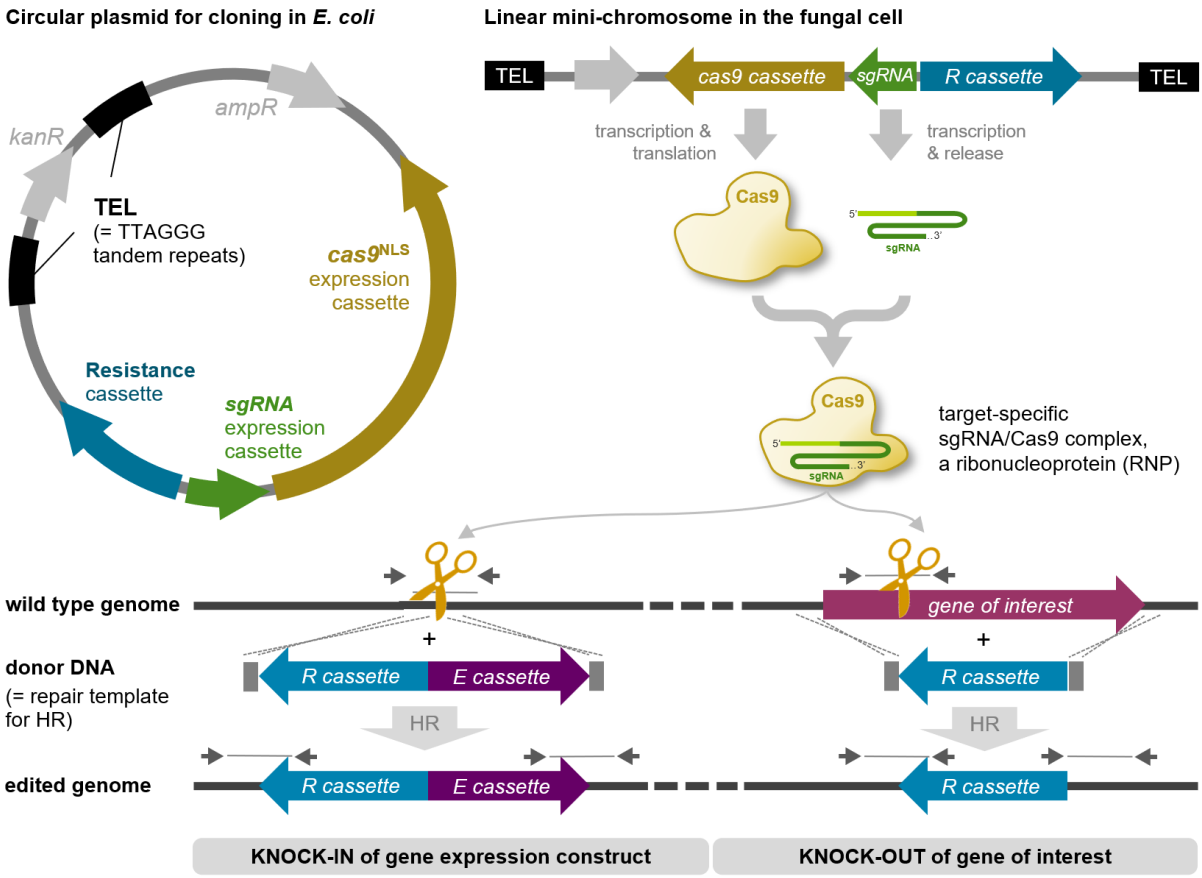

Figure 6. Proposed strategy for transient plasmid-based CRISPR/Cas9 in *M. anisopliae* using telomer-containing plasmids.

Competent blastospores are co-transformed with (i) a circular target-specific sgRNA- and Cas9-delivering CRISPR plasmid and (ii) donor DNA for the targeted mutation (insertion/deletion) of the site of interest by the endogenous homology-directed repair system (HR). The considered pTEL-based plasmid exists as circular DNA in bacterial cells when selective pressure (ampR, kanR) is applied, and linearizes upon uptake into fungal cells and is there maintained as centromere-free mini-chromosome as far as selective pressure on the conferred resistance is applied. Thus, the mini-chromosome can be expelled from the cell by repeated transfer of the edited strains to non-selective medium. The pTEL-derived CRISPR plasmids contain the following components for expression in the fungal cell: a resistance cassette (baR/suR) for selection in *M. anisopliae*, a cassette for expression of target-specific sgRNA (variable 20-bp-long protospacers fused to sgRNA scaffold), and a cassette for the expression of the Cas9-NLS (nuclear localization signal) fusion protein. After co-transformation of the blastospores, the CRISPR components are produced and form the active target-specific RNP, that introduces doubles strand breaks (DSBs, indicated as scissors) in the genomic sites of interests. Donor DNA comprising a resistance cassette and optionally further genes for expression in *M. anisopliae* contain short sequences (~75 bp) homologous to the targeted genomic locus (dark grey bars) and serve as repair template for homologous recombination. A double cross-over event (dashed lines) then results in the exact insertion or deletion of the DNA sequence between the two stretches of

homologous sequences, which can be verified by diagnostic PCR by combining primers (grey arrows) binding up- and downstream of the modified locus with those binding in the integrated sequence such as in the resistance (R) and/or expression (E) cassette.

4. Conclusions and perspectives

The possibility to manipulate the genome of an organism allows for studying gene functions and regulatory networks as well as for elucidating the relevant factors for symbiotic or pathogenic interactions. We are interested in understanding host responses in the termite *R. flavipes*, specifically when individuals are infected by the fungus *M. anisopliae*. By genetically manipulating the genome of the fungus, we would like to generate strains lacking virulence factors such as destruxins and strains stably expressing green or red fluorescence for monitoring the invasive growth within the host and thus understanding the host's immune defences.

Here, we report on a reliable protocol to transform blastospores of *M. anisopliae* with linear DNA conferring resistance to the herbicides GFS and CME. The method is straightforward and lacks laborious and time-consuming steps. Yet, only transformations of freshly prepared competent blastospores were successful. However, adopting the protocol for storage of competent *S. cerevisiae* cells at -80 °C (Gietz & Schiestl, 2007) may allow for the transformation of deep-frozen/thawed blastospores of *M. anisopliae*, which would further simplify the transformation method. In this pilot study, transformations were carried out for the strain DSM 1490 only but both strains DSM 1490 and ARSEF 549 were studied regarding their sensitivity towards GFS and CME and it is assumed that the same transformation procedure can be used for strain ARSEF 549. Notably, the genome sequence for the latter strain is only available (*M. anisopliae* Genome List - NCBI), and that further genetic engineering for studying the specific traits of strain DSM 1490 will also require a sequenced genome. Generally, the blastospore method eases genetic manipulation of *M. anisopliae*. This system would facilitate studies on gene functions and genetic improvements of relevant factors for pathogenic interactions.

The used baR and suR cassettes were fused to a *gfp* expression cassette. As the constructs did not contain homologous sequences to the *M. anisopliae* genome, it is assumed that the integrations occurred ectopically due to random double strand breaks (DSB) and subsequent repair by non-homologous end-joining (NHEJ) resulting in transformants containing the expression constructs in different genomic regions. Strong GFP fluorescence was detected in four transformants, in their conidia, blastospores and hyphae, and when they colonized the host, demonstrating that the used components of the resistance and expression cassettes (resistance genes from different sources with unchanged codon optimization, *B. cinerea* codon-optimized *gfp*, regulatory elements P*trpC*, P*oliC*, T*niaD* and T*gluc*) are functional and enabling significant expression. These observations are highly appreciated as an available large set of modular cloning vectors, initially designed for *B. cinerea* and extended for

application in *K. petricola* (Erdmann et al., 2022a; Schumacher, 2012; Voigt et al., 2020), can be used for advanced genetic engineering of *M. anisopliae*.

Ectopic integrations of expression constructs may cause undesired additional effects, as endogenous genes can be disrupted, or once they are inserted, exogenous genes are not sufficiently expressed. Thus, the targeted integration of expression constructs resulting in independent transformant with an identical genetic background is beneficial. Also, the generation of knock-out mutants by gene replacement relies on the endogenous program of homology-directed DNA repair which is initiated when a repair template – stretches of sequences homologous to the genome – is available and can be used to fix an occurring DSB. The frequency of homologous recombination events i.e. double cross-over events leading to targeted insertion or deletion (**Figure 6**) is usually low but can be drastically increased by inserting DSB in the targeted sequence using the RNA-guided endonuclease Cas9 [CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 system (Doudna & Charpentier, 2014)].

Generally, two strategies for transiently delivering Cas9 and target-specific single guide RNA (sgRNA) to the nuclei of fungal cells exist: (1) DNA sequences encoding for target-specific sgRNA and the Cas9 can be introduced into the cell for expression and *in-vivo* assembly of the sgRNA/Cas9 complex (plasmid-based CRISPR/Cas9), or (2) sgRNA is synthesized, complexed *in vitro* with the purified Cas9 and the assembled ribonucleoprotein (RNP) is introduced into the cell (RNP-based CRISPR/Cas9) (Schuster & Kahmann, 2019). Both delivery strategies work well for Ca^{2+}/PEG-mediated transformation of protoplasts, but the large RNP cannot pass the cell walls of the blastospores. This issue was solved in *B. bassiana* by generating an uracil-auxotrophic recipient strain by *Agrobacterium*-mediated transformation constitutively expressing Cas9. *In vitro* synthesized target-specific sgRNAs and donor DNA with the selectable auxotrophic marker are subsequently introduced into the recipient strain by blastospore-mediated transformation (Chen et al., 2017). As we would like to avoid off-target effects due to constitutive expression of *cas9* and auxotrophic markers, we aim at developing an efficient plasmid-based CRISPR/Cas9 methodology for *M. anisopliae* that is compatible with blastospore-mediated transformation, any *M. anisopliae* strain, and multiplexing by expressing *cas9* and multiple target-specific sgRNA from a single plasmid (**Figure 6**). For this, we currently design optimized CRISPR plasmids that have an extended half-life in the cells of higher Ascomycetes in which the AMA1 self-replicating sequence of *A. nidulans* does not properly function. The strategy includes the combination of the well-working ribozyme-sgRNA, tRNA-sgRNA cassettes and *cas9* expression cassettes from the AMA1-containing CRISPR plasmids (Nødvig et al., 2018; Nødvig et al., 2015) with the pTEL-containing vector backbone. The latter plasmid contains a pair of telomeres i.e. two ~550-bp-long stretches of 'TTAGGG' tandem repeats. They can be efficiently transformed into filamentous fungi and replicate there autonomously as centromere-free mini-chromosomes, but they are rapidly lost in the absence of selection pressure (Barreau et al., 1998). Hahn and co-workers cloned several derivatives including pTEL-START with a multiple cloning site (not published) of the

first telomere vector pFAC1 (Barreau et al., 1998) for using pTEL-expressed resistance genes for selection on CRISPR/Cas9-edited strains in the multinucleate species *B. cinerea*. The performed experiments confirmed the linearization of pTEL-Fen (with a fenR cassette) in *B. cinerea*, and its loss after stop of selection with fenhexamid (Leisen et al., 2020). Pilot experiments showed that the transformation of *M. anisopliae* DSM 1490 with the recently cloned plasmids pTELP and pTELS containing the baR and suR cassettes between the TEL sequences yields GFS- and CME-resistant transformants in which the introduced plasmid can be detected by diagnostic PCR (not shown). In the meantime, the first pTELP-based CRISPR plasmids containing the *cas9* cassette as well as sgRNA for targeting one or both genes encoding key enzymes (polyketide synthases) of pigment synthesis in *M. anisopliae* have been cloned (not shown). Their introduction together with donor DNA (repair templates with homologous sequences to the respective genes) by blastospore-mediated transformation will result in non-pigmented mutants given that the CRISPR components are sufficiently expressed from the mini-chromosome, that the sgRNA is properly released from the transcripts, and that the encoded Cas9-NLS fusion protein enters the nucleus for introducing DSB at the desired genomic sites.

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Supplementary material

Supplementary Table S1. Overview on frequently used selection marker systems in fungi.

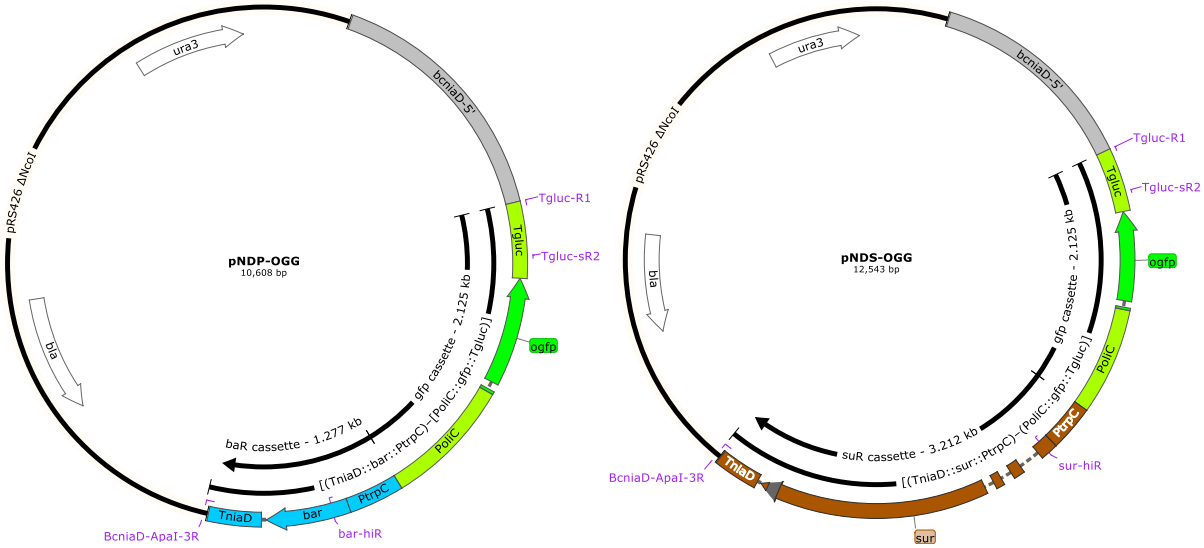
Agent/ R gene	Toxic compound			Resistance		
	Compound	**Target**	**Producer**	**Gene**	**Source**	**Mechanism**
HYG/ *hph*	Hygromycin B (use in R&D only)	Protein synthesis	*Streptomyces hygroscopicus*	*hph* (hygromycin B phosphotransferase)	*Escherichia coli*	Detoxification by phosphorylation
NAT/ *nat1*	Nourseothricin (use in R&D only)	Protein synthesis	*Streptomyces* spp.	*nat1* (nourseothricin N-acetyltransferase)	*Streptomyces noursei*	Detoxification by acetylation
GEN/ *nptII*	Geneticin (G418) (use in R&D only)	Protein synthesis	*Micromonospora rhodorangea*	*nptII* (neomycin phosphotransferase)	*Escherichia coli* (Tn5)	Detoxification by phosphorylation
PHLEO/ *ble*	Phleomycin (use in R&D only)	DNA	*Streptomyces verticillus*	*ble* (bleomycin-binding protein)	*Streptoalloteichus hindustanus*	Detoxification by binding
FEN/ *erg27**	Fenhexamid (fungicide Teldor®)	Sterol synthesis	chemical synthesis	*fferg27* (= insensitive variant)	*Fusarium fujikuroi*	Replacement by insensitive target
CME/ *sur*	**Chlorimuron ethyl** (herbicide Classic®)	Acetolactate synthase	chemical synthesis	***sur*** (= insensitive variant)	*Magnaporthe oryzae*	Replacement by insensitive target
GFS/ *bar*	**Glufosinate ammon.** (herbicide Basta®)	Glutamine synthetase	chemical synthesis	***bar*** (phosphinothricin N-acetyltransferase)	*Streptomyces hygroscopicus*	Detoxification by acetylation

Supplementary Table S2. Oligonucleotides used in this study.

Name	Sequence (5' to 3')	Features (5' to 3')
T*gluc*-R1	GATCTTGTTGGGGGGAAGGGGTTGTCAAATC	*Botrytis cinerea* T*gluc*
***BcniaD*-3R**	gcggataacaatttcacacaggaaacagcgggccc–GCATTGGATTAATAATTGTTGCTAAGC	pRS426 – *Botrytis cinerea* T*niaD*
T*gluc*-sR2	CCGCCCTCTTTTGTCTTCCGC	*Botrytis cinerea* T*gluc*
***bar*-hiR**	GAAGTTGACCGTGCTTGTCTCGATG	*Streptomyces hygroscopicus bar*
***sur*-hiR**	GCTTGAGAGTCGAGATGGTTCGTG	*Magnaporthe oryzae sur*
NS7	GAGGCAATAACAGGTCTGTGATGC	18 s rDNA (small ribosomal subunit)
LR5	TCCTGAGGGAAACTTCG	28 s rDNA (large ribosomal subunit)

	µg/mL hygromycin B						µg/mL nourseothricin						Cell number	µg/mL geneticin						µg/mL fenhexamid					
	0	150	300	450	600	750	0	150	300	450	600	750		0	150	300	450	600	750	0	10	20	30	40	50
M. anisopliae 549	+	+	+	+	+	+	+	+	+	+	(+)	(+)	5×10^3	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)	1×10^4	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)	5×10^4	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)	1×10^5	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+
M. anisopliae 1490	+	+	+	+	+	+	+	+	+	+	(+)	(+)	5×10^3	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)	1×10^4	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)	5×10^4	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)	1×10^5	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	(+)	(+)		+	+	+	+	+	+	+	+	+	+	+	+

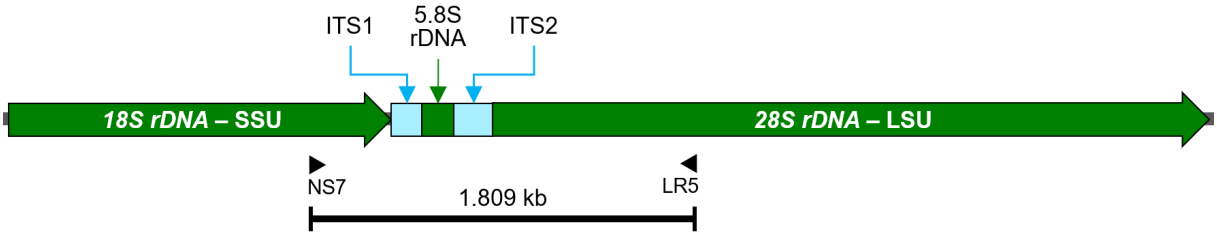
Supplementary Figure S1. *M. anisopliae* strains ARSEF 549 and DSM 1490 are insensitive to four frequently used selective agents. Two wells (technical replicates) of 96-well microtiter plates per strain were filled 225 µl blastospores (2.2×10^4, 4.4×10^4, 2.2×10^5 and 4.4×10^5 cells/mL SDNG) per well and 25 µl serial dilution stock concentration of 100 mg/mL H_2O of HYG (hygromycin B) and NTC (nourseothricin) and 50 mg G418 (geneticin)/mL H_2O and 5 mg FEN (fenhexamid)/mL EtOH. Growth was evaluated after 16 days of incubation at 25 °C in constant darkness: + good growth, – no growth, (+) poor growth.



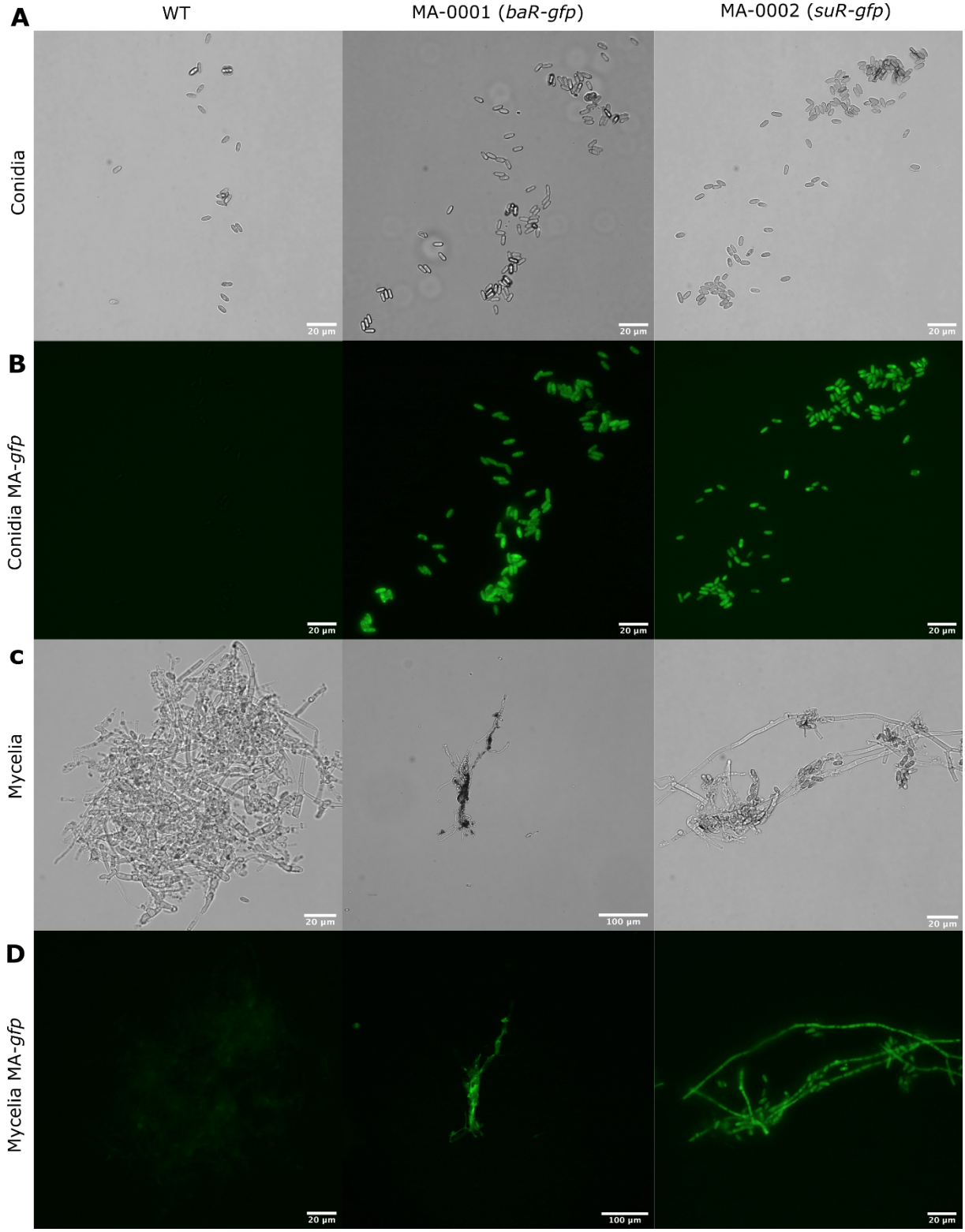
Supplementary Figure S2. pNDP-OGG and pNDS-OGG used for the transformation of *M. anisopliae* blastospores in this study.

The two plasmids derive from the pNXR-XXX series of cloning vectors which have the same modular structure and were primarily designed for usage in *Botrytis cinerea* (Schumacher, 2012). The plasmids contain a resistance cassette and an expression cassette in opposite orientation that are flanked by the 5' and 3' noncoding regions of *bcniaD* (encoding the nitrate reductase) for facilitating targeted integration into the respective gene locus of *B. cinerea*. The 3' noncoding region represents the terminator of *bcniaD*, and thus function as terminator (T*niaD*) of the resistance gene. P*trpC* and P*oliC*

are constitutive promoters from *Aspergillus nidulans*. The contained GFP-encoding gene was optimized for usage in *B. cinerea* (adapted codon usage, addition of an *B. cinerea*-derived intron in the 5' region) and found to mediate robust GFP fluorescence in several other fungi as well. The vector backbone pRS426$^{\Delta Ncol}$ contains *bla* and *ura3* for selection of plasmid-carrying clones in *Escherichia coli* (resistance to ampicillin) and *Saccharomyces cerevisiae* (prototrophy on uracil-lacking medium), respectively. Structurally identical plasmids (pNDR-OGG) are available with genes mediating resistance to hygromycin [H, *hph*], nourseothricin [N, *nat1*], phleomycin [B, ble] (Schumacher, 2012), fenhexamid [F, *fferg27*] (Cohrs et al., 2017), glufosinate ammonium (GFS) [P, *bar*], and chlorimuron-ethyl (CME) [S, *sur*] (Erdmann et al., 2022).



Supplementary Figure S3. The nuclear rDNA operon of *M. anisopliae* 549.
The sequence was retrieved from Ensembl [*Metarhizium anisopliae* ARSEF 549 supercontig scaffold_56 MAN_1.0]. ITS1/2 – internal transcribed spacer 1/2, SSU – small ribosomal subunit, LSU – large ribosomal subunit. NS7 (White et al., 1990) and LR5 (Hopple Jr & Vilgalys, 1994) are standard primers used for ITS barcoding and thus are not fungal specific. They were used in this study for DNA control PCRs.



Supplementary Figure S4. Verification of the expression of enhanced green fluorescence protein (*gfp*) gene in *M. anisopliae* 1490.

A: Brightfield images of *M. anisopliae* conidia from wild type (WT) and *gfp* transformants under a fluorescence microscope. **B:** Detection of gfp fluorescence in conidia from Wt and *gfp* transformants under a fluorescence microscope stably expressed in the fungal cell. **C:** Brightfield images of *M. anisopliae* mycelia from wild type (Wt) and *gfp* transformants under a fluorescence microscope. **D:** Detection of *gfp* fluorescence in mycelia from Wt and *gfp* transformants under a fluorescence microscope was stably expressed stably expressed in the fungal cell.

GENERAL DISCUSSION

In this thesis, we investigated the social immune defence in the subterranean termite *Reticulitermes flavipes* upon challenge of the immune system either with the native entomopathogenic fungus *Metarhizium anisopliae* or the novel entomopathogenic bacterium *Pseudomonas entomophila*. Since pathogens have acted as strong selection pressures on termites, shaping the evolution of sophisticated defence mechanisms (Schmid-Hempel, 1998), termite colonies provide a unique opportunity to study host–pathogen interactions and animal immunity as they have evolved collective immune defences or social immunity that complement the innate immune response of individual colony members (Cremer et al., 2007).

Once pathogens are inside the colony, they can be rapidly spread and impact colony health (Cremer et al., 2007). In particular, entomopathogenic fungi greatly profit from the large density of susceptible individuals with genetic homogeneity, because transmission opportunities and the chance to prevail is extremely increased. This is true for colonies of the termite, *R. flavipes*. Therefore, we investigated distinct aspects of the host–pathogen system between *R. flavipes* and *M. anisopliae*, the step-wise infection dynamics as well as the importance of the link between physiological and behavioural adaptations to mitigate the occurrence and transmission of infectious diseases. Furthermore, we tackled how termite social immunity can be applied against novel *P. entomophila* infections or whether it is specific against *M. anisopliae* infections. We focused on the mechanistic underpinnings of the care−kill dichotomy (Cremer & Sixt, 2009) directed towards differently infected individuals. The interaction between an infected nestmate could lead to: 1) special care to cure the infected individual attempting to prevent the onset and spread of infections and alternatively 2) when the infection agents break through this early defence it might be adaptive to simply eliminate infectious individuals from the colony to prevent replication and transmission of disease. We identified two major defence mechanisms of the care-kill dichotomy of termites that interrupt the pathogen's life cycle; allogrooming and cannibalism, and the potential chemical signal(s) responsible for triggering elimination behaviours to protect the colony from fungal and bacterial diseases.

In Chapter I, we discovered that elimination not only of internally challenged but also sick individuals is targeted to decrease the transmission risk of an infection within the colony and improve its survival. By injecting *M. anisopliae* blastospores directly into the hemocoel of individuals to cause an internal infection, we removed the pathogen as a direct cue for responding nestmates. We found that collective defences towards injected individuals differ according to the severity of pathogen challenge, that is even an inactive fungal challenge (injection of dead blastospores) resulted in near complete elimination of the injected individual, via cannibalism, but at a significantly slower rate than in a severe active fungal challenge (injection of viable blastospores). Moreover, despite the absence of external pathogens, we were able to detect elevated rates of grooming at early time points following

infection (2 and 8 hours), and although grooming rates were lower in comparison to previous studies employing conidia on the termite cuticle (Chouvenc et al., 2009a; Davis et al., 2018; Liu et al., 2019b; Myles, 2002; Rosengaus et al., 1998a; Yanagawa et al., 2012; Yanagawa & Shimizu, 2007) our results are in line with Davis et al. (2018) that demonstrated that once the pathogen has penetrated the cuticle, and the infected termite can no longer be cured, nestmates still showed a large grooming response but that this was likely caused by direct interactions with external pathogens, which we hypothesize was the main driver eliciting an intense grooming response (Davis et al., 2018). This intense grooming may also ultimately lead to cannibalism to prevent sick individuals from proliferating disease. The persistent nature of termite grooming may act as a constant vigil, helping to continuously protect from disease spreading throughout the colony (Aguero et al., 2020). However, we argue that the effect of grooming for the host is limited to external infections. Nonetheless, it may also play a vital role during the initial inspection phase of an interaction with an internally infected individual.

In contrast, cannibalism was particularly evident when active fungal injected individuals became visibly ill and when they were close to death, but cannibalism is still observed when individuals were not necessarily terminally ill or when the challenge did not itself always result in host death (early time points after active or inactive fungal challenge). More specifically, all termites injected with dead blastospores were cannibalized, although this challenge was associated with a high percentage of recovery (~60%) and even though dead blastospore injected termites did not display severe signs of sickness by any time point (2, 8, 12 and 15 hours). A similar pattern was observed when termites were injected with a toxin- and cells-depleted inactivated pathogen solution, showing that even subcellular fungal components lacking active toxins are enough to elicit cannibalism. This indicates that the threshold for cannibalism occurs at an early-stage during the internal infection specifically before pathogen viability or even terminal disease status is determined, similar to the internal immune system in the body that rapidly targets elimination of infected cells once an infection occurs (Cremer & Sixt, 2009). Cannibalism rates towards ill individuals gradually increased, as the disease progressed. Indeed, injected individuals with viable blastospores appeared healthy at 8 hours but moribund at 15 hours, time point in which cannibalism was significantly increased, due to the large, exhibited sickness cues affecting the course of fungal infection. This advance-stage of infection is linked on one side to evasion of host innate immune responses and production of toxins by the pathogen (Chouvenc et al., 2009a; Hänel, 1982; Iwanicki et al., 2020; Roberts, 1966; Syazwan et al., 2021) but on the other host moribundity is linked to toxin release after an effective breach of the fungal hyphae into the hemolymph ultimately leading to host death (Gillespie et al., 2000). The chance to survive of an infected individual once *M. anisopliae* enter the body cavity as well as the chance of recovery of an ill individual is low to non-existent, which should favour early “elimination” of the infection from the colony via cannibalism to prevent the disease from spreading, and that is what we observed. These results complement previous findings from fungus-exposed individuals that were cannibalized when termites were moribund (Davis et al., 2018; Rosengaus & Traniello, 2001) but with higher rates of cannibalism when termites were near death (Davis et al., 2018). This is consistent with

Yanagawa et al. (2011b) that reported that cannibalism is activated by signals released after infection (Yanagawa et al., 2011b), exactly when an internal-stage in *M. anisopliae* infection has been reached (Davis et al., 2018).

By injecting dead blastospores, we were able to partially separate pathogen replication and toxin release within the host hemolymph from subsequent host death. Here, we showed that nestmates could not discriminate between the viability of an internal pathogen, nor its true moribundity effect, meaning that they generally detect individuals that have an activated innate immune system (activation of specific signalling pathways) (Liu et al., 2019a) through the perception of a trigger signal possibly of behavioural or chemical nature (Konrad et al., 2012). Alteration of the cuticular profile of hosts most probably results from the infection process and elimination behaviours could be displayed when changes in the cuticular profile exceed a given threshold (Leclerc & Detrain, 2016). In line with this, we determined that termites might have evolved chemical means of identifying internal infections through changes in the cuticular hydrocarbon (CHC) profile and adjust their behaviour in response. Alterations in the CHC profiles were found in individuals injected with viable but not with dead blastospores. More specifically, we detected significant increases in four exclusively methyl-branched CHCs at 15 but not at 12 hours post injection with viable blastospores whereas there was no such increase in control-injected individuals. Indeed, we observed a clear effect of *M. anisopliae* infection on four alkanes, 11-MeC35, 12-MeC36, 11-; 13-MeC37, and 11,15-DiMeC37, which were found in higher relative amounts. Although an increase in these compounds was also clearly visible in individuals injected with dead blastospores, significant differences were not found. Coincidently, at 15 hours' time point we observed the highest amounts of cannibalism as well as the onset of visible signs of sickness. These compounds might be encoding and conveying a potential chemical cue associated with internal immune activation or infection status. Such cues might have evolved in termites because they alert their nestmates to the presence of a developing infection that will harm the colony if a disease outbreak arises (Aguero et al., 2020). Overall, these results reveal that changes in termite chemical profile can be directly linked to an immune response developed by the host after infection, but also fungal growth inside the insect body cavity could modify the odour of the infected termite, similar to findings in honeybees (Baracchi et al., 2012; Conroy & Holman, 2021; Hernández López et al., 2017; Nielsen & Holman, 2012; Richard et al., 2008) and ants (Pull et al., 2018; Csata et al., 2017) that produced a different blend of CHCs after infection. Likewise, the increased amounts of long-chained mono- and di-methyl-branched alkanes over time following viable blastospore injection, as well as a higher trend in quantities of these compounds in dead blastospore injected termites suggests that they may play an important role in eliciting cannibalism. Further research is needed to empirically test the causal role played by CHCs in triggering social immune responses, in which individual or blend compounds, behaviours, or mixtures thereof might be the actual trigger.

Dead blastospores still bear components of cell wall that could activate the immune system of the host. It has been shown that immune priming can occur after injection of dead cells,

which may trigger an immune response due to the presence of PAMPs (Wu et al., 2014). However, dead blastospores do not produce toxins, which may also elicit an immune response acting as a "danger" signal, or by a negative influence on host's cells as a damage signal (Ming et al.,2014). Injection of heat-killed blastospores of the fungus *Beauveria bassiana* appear not to prime the insect immune system (Vertyporokh et al., 2020) but instead host injected with fungal filtrates can become moribund or died (Roberts, 1966). It is likely that virulence factors take part in immune priming. If that is the case, the faster cannibalism response to viable blastospore-injected termites may be related to the presence and active synthesis of destruxins acting as virulence factors. Some research examining the interaction between sickness cues and social immunity (Yanagawa et al., 2011b; Davis et al., 2018; Pull et al., 2018; Swanson et al., 2009; Al Toufailia et al., 2018) implied indirectly the recognition of fungal virulence factors. Although the recognition mechanism of virulence factors requires further examination, toxins are indeed able to induce social immunity (Liu et al., 2019b). It is, however, safe to claim that these results await the next step in the research of the termite-entomopathogen interactions, specifically interactions between all these infection components with the termite immune system and validate how toxins may be a driving force in the evolution of social immunity.

We found that our *M. anisopliae* strain produces five different types of cyclic peptide toxins, destruxins (A, B, C, D and Ed). Destruxins play an important role in pathogenesis (Kershaw et al., 1999) and secretion of these toxins during infection has been shown to result in more rapid host death compared to strains that do not produce these toxins (Suzuki et al., 1971; Kershaw et al., 1999; Rustiguel et al., 2018). In this regard, *M. anisopliae* can have two different infection strategies to kill the host referred to as toxin and growth strategies (Vega et al., 2012). Specialization is thought to be associated with a growth strategy (involves growing within the living host and produces little or no toxins) and opportunism with a toxin strategy (Roberts & St. Leger, 2004). Destruxins function directly to kill the host or support the infection process indirectly by suppression of the host immune response (Dong et al., 2009). Therefore, the toxin strategy present in our *M. anisopliae* strain might also partly contribute to the lower survival of viable blastospore-injected termites in a colony as seen in Chapters I and II. However, these pathogenic strategies might influence both the hosts' immune system and their behavioural responses by changing or delaying them. Therefore, a better understanding between these infection strategies and disease defences from hosts holds promise to the development of *Metarhizium* as biocontrol agent against insect pests (Zimmermann, 1993). To date only one study has evaluated the mechanism of action of destruxins on termite hemolymph cells in which a higher level of intracellular calcium was found. This suggest that destruxins destroyed the calcium channels on the cell membrane of hemolymph, which facilitated the influx of $^{45}Ca^{2+}$ outside the hemolymph (Dong et al., 2009). Nevertheless, more research is needed to understand termite immune responses modulated by destruxins.

Together with the significantly lower survival rates observed in both inactive and active fungal challenge as compared to the controls, we hypothesize that: a) an internal fungal challenge

triggers cannibalism as a mechanism to eliminate individuals before they enter a terminal disease state, so cues responsible of the activation of this behaviour are generated during early phase, specifically prior to the onset of moribundity, b) moribundity is linked to virulence factors and they might play a key role in the intensity of the cannibalistic response, c) despite that the pathogen was effectively dead, termites cannot accurately identify the viability or associated moribundity of an internal *M. anisopliae* challenge but they can detect individuals that have an activated immune system, d) the fungal pathogen deploys a multi-component infection strategy, the toxic strategy, to suppress the innate immune system, kill the host and complete the infection cycle (Gillespie et al., 2000), and e) alteration of the cuticular profile in immune-challenged individuals could play a role in triggering elimination behaviours.

Beyond the role of social immunity to fight fungal infections, social immunity can also function when fighting bacterial infections. *Pseudomonas entomophila* is a potent entomopathogenic bacterium. In our study, we found that *P. entomophila* can kill members of *R. flavipes*. Hence, in Chapter II, we explored the interaction between *R. flavipes* and *P. entomophila* and the mechanisms underlying the social immune response against bacterial infections as well as directly compare collective behaviours in response to different entomopathogenic challenges – one fungal and one bacterial – of varying intensities. We found that collective behaviours in *R. flavipes* vary according to the infection type and severity, but specific defence mechanisms may be employed against fungal infections. By injecting either viable and dead cells of *P. entomophila* or viable and dead blastospores of *M. anisopliae* we revealed a consistent behavioural pattern with the one observed in Chapter I when an individual with an established internal *M. anisopliae* infection is present in the colony. We observed low rates of grooming which were progressively replaced by cannibalism and therefore an overall lower survival probability was detected for both bacterial and fungal infected individuals as compared to controls. Because infected individuals pose a direct risk of epidemic outbreak in the colony, a rapid behavioural response or more effective strategies are expected. Comparisons between highly lethal doses of viable-*P. entomophila* and *M. anisopliae* blastospores (100% mortality) exhibited higher amounts of cannibalism leading to the rapid elimination of focal termites, independent of pathogen type used. Contrary, an interesting difference was observed when termites challenged with an intermediate dose (40% mortality) of viable-*P. entomophila* and dead-*M. anisopliae* blastospores were compared, in which the cannibalism response was predominantly evident in all individuals injected with dead blastospores as opposed to an equivalently bacterial infection. Differing in the ant *Camponotus pennsylvanicus*, cannibalism was directed towards individuals that were infected with both heat-killed and live *Serratia marcescens* (Rosengaus et al., 2013).

Based on these results, we not only argue that collective responses can be transferable to diverse and potentially novel infection threats like *P. entomophila* but also that the effectiveness of these responses, particularly cannibalism, might rather be more pathogen-specific. Yanagawa et al. (2011a) found that *M. anisopliae* caused more aggressions and cannibalism in termites than other genera of entomopathogenic fungi (Yanagawa et al.,

2011a). Though, very specific responses towards infected nestmates might depend not just on the type of pathogen and infection intensity of a specifically infected individual but also on the health status of the entire colony (Biganski et al., 2018). This strengthens the idea that nestmates are capable of detecting specific chemical or other triggers for *M. anisopliae* blastospores or internal fungal pathogen presence suggesting that moribundity is not a necessary cue for cannibalism (Davis et al., 2018) as observed in Chapter I. Cannibalism as the kill-component of termite social immunity (Cremer and Sixt, 2009) appears to be associated with internal immune challenge affecting host–pathogen dynamics and it re-enforces the view of a colony as an integrated organism. Elimination of infected kin to protect the colony has also been observed in ants and honeybees (Pull et al., 2018; Rosengaus et al., 2013; Spivak & Gilliam, 1998; Swanson et al., 2009; Al Toufailia et al., 2018; Biganski et al., 2018).

The strongest cannibalism response to 40% mortality blastospore-treated termites, suggest that inactivate or low doses of pathogens can lead to significant immune stimulation and potentially also trigger changes to cuticular chemistry. For instance, infection with killed bacteria can elicit an immune response, including the increase of AMPs. This is consistent with Keesey et al. (2017) where infection with heat-killed *P. entomophila* resulted in the production of two different AMPs including Drosomycin and Diptericin as well as a smaller but significant increase in fatty-acid-derived pheromone production in *Drosophila* (Keesey et al., 2017). Similarly, Cathepsin D was found to occur in higher amounts in the trophallactic droplets of ants after injection of heat-killed *S. marcescens* or lipopolysaccharides (LPS) (Hamilton et al., 2011b). Bacterial cell wall components (LPS and peptidoglycan) are known to be highly immunogenic (Gallagher et al., 2018). Also, injection of honeybees with freeze-killed *E. coli*, caused the activation of the humoral immune pathways (Richard et al., 2012). In termites, Rosengaus et al. (1999) found that injection of glutaraldehyde-killed *Pseudomonas aeruginosa* generated an immune response that conferred higher survival to subsequent challenges with alive *P. aeruginosa* and induction of proteins appeared to differ in bacteria-injected and fungus-exposed termites (Rosengaus et al., 1999), suggesting specificity in the immune response. Fungus-specific immune responses in ants were found to occur (Konrad et al., 2012). Similarly, specific immune upregulation after Gram-negative bacterial and fungal infection is known to occur in *Drosophila* (Lemaitre et al., 1997). Moreover, inoculation of low doses of pathogen might enhance pathogen resistance in eusocial insects (Ezenwa et al., 2016). Indeed, we observed that termites reacted strongly to *M. anisopliae* infections even at lower level specifically when the pathogen was inactive as seem in Chapters I and II. Since injection of blastospores increase the PO levels in insects (Gillespie et al., 2000), this could also play a role in signalling and therefore influence the response in termites.

Although the different infection types (bacterial and fungal) caused somewhat similar changes in termite behavioural responses, we also found evidence for the hypothesis that social immunity in termites leads to a greater protective effect for fungal over bacterial infection. Aside from a likely evolutionary old association with soil-dwelling entomopathogenic fungi, a proposal that the distinct infection modes of bacterial and fungal pathogens could also

underly these differences. Bacterial infections typically occur via oral uptake, indicating that bacteria-infected individuals will not carry transmissible spores on their cuticle, as is the case for entomopathogenic fungi (Konrad et al., 2012). Richard et al. (2012) suggested that behavioural changes might be adaptive: it is possible that social insects have evolved to "match" the strength of the signal to the virulence of the pathogen (Richard et al., 2012), and thus infection with entomopathogenic fungi would elicit a greater change in the behavioural responses of nestmates than with bacteria. Overall, in Chapter II, we confirmed how termites can generate cross-protection against other pathogens like *P. entomophila*, indicating that social immunity can also function against bacterial infections (Cremer et al., 2007), albeit less efficiently.

Social immunity may play an important role in fighting manifold pathogens present within a colony (Cremer et al., 2018). *Pseudomonas entomophila* infections cause insects to rapidly succumb despite the induction of innate immune responses, indicating that this bacterium has developed specific strategies to escape the insect immune response (Liehl et al., 2006; Tetreau, 2018) similar to *M. anisopliae* that camouflages its antigenic β-glucans via collagen-like protein (Wang & St. Leger, 2006) and releases toxins or virulence factors during host infection (Iwanicki et al., 2020; Schrank & Vainstein, 2010) as confirmed in Chapter I. These virulence factors or subcellular components might be detected by nestmates and may play a role in signalling consequently causing a robust collective defence as observed for viable blastospore-injected termites in Chapters I and II that were highly cannibalized to reduce the ability of the infection to progress throughout the colony. However, how social immunity directly monitors fungal toxins and discriminates fungal toxins from bacterial toxins needs further examination. A possibility for experimentally checking the role of toxins in the regulation of social immune response would be to compare behaviours towards a pathogenic but non-toxic bacterium. This study is the first to identify the toxicity of *P. entomophila* against *R. flavipes* (Chapter II), nevertheless the specific components and mechanisms of this toxicity are unclear. Research has shown that the pathogenesis of *P. entomophila* is multi-factorial due to the action of metalloprotease, monalysin pore-forming toxin, insecticidal toxin-like proteins, secondary metabolites or hydrogen cyanide (Liehl et al., 2006; Vodovar et al., 2006; Opota et al., 2011). However, the active components of *P. entomophila* that are toxic to termites have not been identified.

Comparisons between high lethal bacterial and fungal infections showed that grooming was only higher at 15 hours in bacterial infections, which corresponded to the earlier onset of cannibalism in response to high lethal fungal infections. However, lower rates of grooming are correlated with both intermediate bacterial and fungal infections, which indicates that a certain threshold of infective cells or stimuli must be reached for detection and inspection by nestmates. Overall, the low rates of grooming observed in Chapters I and II might only indicate early pathogen detection, as there is nothing to be mechanically removed from the cuticle. Early detection of pathogens should also help to prevent disease spread. Therefore, termites might interact with bacterial or fungal challenged individuals through grooming to upregulate

their immune system leading to a “protective immune stimulation” otherwise known as “active immunization”, which can improve their resistance to pathogens (Traniello et al., 2002; Liu et al., 2015). However, direct detection of *M. anisopliae*-blastospores or bacteria by nestmates seems unlikely, but injection of both could trigger the infected individual to produce chemical cues after activation of the innate immune system as confirmed in Chapter I. As these chemical signals most likely involve modifications of the CHC profile, we able to find differential patterns of cuticular hydrocarbon (CHC) profiles among all treatments, particularly discernible was the profile of termites injected viable blastospores [100%], which by the investigated time point (15 hours) was associated with the highest observed rates of cannibalism. Following an immune challenge, the CHC profile rapidly change (Conroy & Holman, 2021). Accordingly, infection with *M. anisopliae* blastospores and *P. entomophila* may have altered the CHC profiles of individuals in a way that could be detected by nestmates and subsequently trigger elimination behaviours. In line with these results, bacteria-challenged (heat-killed and alive) *Tenebrio molitor* showed differences in immune and fitness traits compared to healthy conspecifics suggesting that *T. molitor* responds to immune-related signals in the social environment that correspond to modifications in the CHC profile during immune stimulation (Gallagher et al., 2018). Therefore, the role of CHCs in host–pathogen interactions is a fascinating feature to prevent pathogens from spreading in the colony (Schuehly et al., 2021). Different immune elicitors stimulate unique responses at behavioural, chemical and molecular levels (Richard et al., 2012); for example, *P. entomophila* injection should activate the Imd pathway and *M. anisopliae* injection should activate the Toll pathway, which could lead to distinct chemical profiles and behavioural responses. Accordingly, we not only observed changes in the chemical profile of individuals injected with fungal and bacterial pathogens but also in the behavioural responses, remarkably at intermediate infection level (Chapter II).

Chemical cues can not only trigger behavioural defences, but also profound alterations at the genetic level (Hernández López et al., 2017). Mechanistic links between the CHC profile and immune status has been demonstrated through changes in the expression of genes that have pleiotropic effects on lipid metabolism/homeostasis, such as those in the Imd pathway (Pull et al., 2018; Richard et al., 2012) as these genes are the biosynthetic precursors of CHCs in insects (Hernández López et al., 2017) leading to distinct behavioural responses. Several genes that may play a role in CHC biosynthesis were identified in honeybees following challenge with Gram-negative bacteria (*Escherichia coli*), which began to produce relatively more unsaturated and shorter-chained hydrocarbons (alkanes) within six hours, and there were concomitant changes in the expression of genes involved in CHC biosynthesis. Alternatively, the expression of a few key genes and/or chemical profile components in bacteria-challenged honeybees triggered significantly behavioural changes (allogrooming and aggression) in nestmates (Richard et al., 2012). Genomic responses to immune stimulation may mediate the collective behavioural changes by orchestrating changes in chemical signalling (Richard et al., 2012). However, no study has yet reported the interaction between CHC profile and genomic responses in to immunostimulation in termites.

In order to maximize fitness of the colony, we should expect eusocial insect individuals to signal honestly about their health status, regardless of disease progression. That is, they should evolve to signal to be cured when contaminated and signal for elimination when infected (Cremer, 2019). In this study, we showed that internal infections with *M. anisopliae* and *P. entomophila* pathogens lead to two different behaviours that are comparable to the care-kill dichotomy of social immunity. However, cannibalism is the main response associated with internal infections that targets elimination of the infected individual regardless of pathogen type and condition as well as disease status. This is in line with Cremer, 2019, who states that once an infection has developed to the point of no return, social immunity switches from a care to a kill strategy. Like in termites, the same selective pressure has driven the evolution of an independently complex behavioural repertoire − destructive disinfection in ants − Hygienic behaviours: uncapping, dropping out to aggressive behaviour (biting, stinging) in honeybees − to eliminate infected individuals early in the infectious cycle and prevent disease transmission (Pull et al., 2018; Spivak & Gilliam, 1998; Swanson et al., 2009; Al Toufailia et al., 2018; Biganski et al., 2018). These processes are functionally equivalent to the elimination of infected cells in a body, which emit a type of "find me, eat me" signal, MHC class I proteins to present antigens to cytotoxic T cells, which then destroy the infected cells (Cremer et al., 2019; Conroy & Holman, 2022). Likewise, the infected individuals have an altered chemical profile that is accurately detected by the nestmates to prevent damage of healthy colony members (Pull et al., 2018; Cremer, 2019; Schuehly et al., 2021). As changes in CHC profiles may provide information about the health status and facilitate social immunity by influencing the behavioural response (Richard et al., 2008; Baracchi et al., 2012). Together, these results suggest that the changes in the CHC profile of immune-challenged termites are important markers for detection of internally infected nestmates, and for responding behaviourally by eliminating them as observed in Chapters I and II. This indicates that the host itself may play a role in signalling its own demise. Thus, the chemical communication system of termites based on cocktails of CHCs may possess other functions than simply intra- and interspecific recognition (Kaib et al., 2004).

The kin selection theory predicts that eusocial insects might have evolved unconditionally altruistic responses to disease (Hamilton, 1964; Cremer et al., 2018; Shakhar, 2019; Conroy & Holman, 2022). In eusocial insects, workers reproduce indirectly by helping relatives with whom they share copies of the same genes, and hence gain only their indirect fitness (Cremer, 2019). Accordingly, if the presence of an individual flips from having a beneficial to a harmful effect on the fitness of its relatives - such as when an individual contracts a lethal infection - signalling for elimination may be an action that indirectly increase its own inclusive fitness by protecting the colony from infection. Thus, ensuring that copies of its genes are passed into the next generation via its relatives (Cremer et al., 2018). But, through kin selection, evolution may have also favoured not only individuals who display such signals but also who can detect them. In agreement with this idea, honeybees that were immune-challenged with LPS left the hive and subsequently died; some individuals were dragged out by nestmates, while others

appeared to leave voluntarily. Also, individuals coated with extracted CHCs from LPS-challenged honeybees were removed from the hive more often than controls, indicating that immune-challenged individuals produce chemical cues that elicit their removal. The authors suggested that sick individuals are removed by nestmates using olfactory cues, putatively because of kin selection to limit the spread of pathogens within colonies (Conroy & Holman, 2022). Likewise, our results imply that termites eliminate infected individuals early in the infection cycle by using chemical cues, likely favoured by kin selection. However, further research is needed to test whether chemical signalling of health status by infectious individuals and detection of sicknesses by healthy individuals has been favoured by kin selection as stated by Conroy & Holman (2022) as well as test whether the chemical changes found in Chapters I and II are the true trigger of social immunity. One could test in a comparative approach using several lineages across the termite phylogeny whether they undergo uniquely strong chemical or behavioural changes following an immune challenge.

Termites have evolved highly complex social interactions with infectious microbes, which consists of recognition, communication, and a combination of multi-layered defence strategies (Liu et al., 2019b). These social interactions can prevent pathogenic infections and flexibly limit disease transmission. Termites are able to rapidly detect fungal and bacterial cues and accurately display several physiological and behavioural disease defences or the cooperation of both to protect the colony. More specifically they can effectively detect and eliminate different established internal infections, potentially through modifications in the CHC profile, even before they become contagious and external sickness cues have appeared. This represents the kill strategy of social immunity and confirms the ability of *R. flavipes* termites to detect pathogens early and anticipate sanitary risks associated with the infection process like sporulation in the case of entomopathogenic fungi to prevent epidemics within their colonies. Thus, pathogen resistance in termites is greatly enhanced due to their collective defence highlighting the importance of social immunity for the success of insect societies and may have even been necessary for the evolutionary emergence of superorganisms.

In Chapter III, we found that GNBP-2 inhibition did not play a role in the rate or intensity of grooming behaviour of either uninfected nestmates or fungus-exposed nestmates. However, the amount of grooming was significantly higher in all *M. anisopliae*-exposed individuals. Thereby grooming is performed adaptively according to exposure risk and it is an effective mechanism to disinfect insect cuticles and clear external pathogens in early-stage infection (Davis et al., 2018; Hamilton & Bulmer, 2012; Liu et al., 2015; Yanagawa & Shimizu, 2007). In addition, ingestion of infectious conidia ultimately ends up in the termite's gut, where are unable to germinate (Chouvenc et al., 2009a). This response can prevent the immune system of individual termites from being overwhelmed by numerous simultaneous infections (Chouvenc & Su, 2010; Chouvenc et al., 2009b) ultimately decreasing their physiological investment in innate immunity (Liu et al., 2019a). Specifically, grooming has been shown to increase after conidia adhesion, becoming even more elevated and intense after conidia germination (Davis et al., 2018), which in turn predicts survival of exposed individuals within

a colony (Rosengaus et al., 1998b; Yanagawa & Shimizu, 2007). This suggest that grooming is primary induced by the presence of fungal pathogen-associated molecular patterns (PAMPs) (Davis et al., 2018; Yanagawa et al., 2011b). Yet, the effect of grooming can be sometimes restricted to the fungal pathogenesis (Clarkson & Charnley, 1996) as the fungus adheres to the termite cuticle and penetrates it within 12-24 hours to reach the host hemocoel (Moino Jr et al., 2002; Neves & Alves, 2004), thus limiting the period at which grooming can actively remove conidia. The limits of grooming set through fungal pathogenesis might also in part explain the lower rates of grooming observed in Chapters I and II when termite colonies face internal infections with *M. anisopliae* blastospores.

Despite the sanitary care towards fungus-exposed individuals, we found in Chapter III that the GNBP-2 is unlikely to be the core mechanism by which termites detect fungal PAMPs, but that its activity can influence other collective behaviours, such as cannibalism once the host becomes sick, possibly via the release of fungal PAMPs from fungal-damaged host cuticle. Cannibalism is displayed once an internal infection has occurred and the host showed signs of sickness, including moribundity (Davis et al., 2018) in which the fungus went through a single-celled blastospores stage. We showed that cannibalism response is positively affected by the β-1,3-glucanase activity of termite GNBP-2. Suppression of the GNBP-2 activity resulted in a significant reduction of cannibalism in fungus-exposed nestmates compared to uninfected nestmates. During cannibalism, these antimicrobial properties in both termite´s saliva and gut contribute to inhibiting pathogen transmission (Chouvenc et al., 2009a; Lamberty et al., 2001). These findings suggest that fungal factors in combination with host-moribundity triggers both grooming and elimination behaviours in termites, as well as GNBP-2 activity can regulate the transition between these states. Although, we have confirmed a link between externally secreted antimicrobial compounds or immune molecules and “care or kill” collective defence in termites, the inhibition of the fungicidal immune enzyme, GNBP-2 has a high impact on the disease resistance mechanism which in turn could affect colony-wide immune competence. The secreted-β-1,3-glucanase activity of GNBP-2 acts as potent external disinfectant and it has been detected on the termite cuticle as well as in nest-construction materials (Bulmer et al., 2009). Because GNBP-2 appears to be essential for defence against *M. anisopliae* before penetration of the host and its inhibition significantly increases the susceptibility to infection (Bulmer et al., 2009; Hamilton et al., 2011a). More specifically, GNBP-2 also plays an important role in signalling the presence of pathogens by acting as immune effector, priming the termite immune system as well as pattern recognition receptor (Bulmer et al., 2009). The obtained results in Chapter III show on one hand that GNBP-2 is effective not only during early-stage infection but also during advance-stage fungal infection and on the other that immunity of termites relies on complex interactions between individual physiological responses and its underlying genetic architecture and social defences, allowing termites to thrive in environments heavily colonized by pathogens.

In Chapter IV, we developed a transformation system based on *M. anisopliae* blastospores that consisted of the integration of *bar* and *sur* genes, conferring resistance to glufosinate

ammonium (GFS) and chlorimuron ethyl (CME) respectively and expression of the green fluorescent protein (*gfp*) into the fungal genome. Competent blastospores were transformed with the amplified *bar-gfp* and *sur-gfp* constructs resulting in resistant colonies when DNA was added. The GFS- and CME-resistant colonies became visible five days after transformation and grew well after eight days. The stability of the GFS- and CME-resistant phenotype was determined by measuring resistance after two serial transfers on SDNG medium containing 250 µg GFS/mL and 50 µg CME/mL. Furthermore, the blastospore-based transformation system developed in this study has successfully been applied to integration of *bar-gfp* and *sur-gfp* foreign genes into *M. anisopliae* genome, resulting in MA-0001 (*baR-gfp*) and MA-0002 (*suR-gf*p) transformants which expressed green fluorescence protein in conidia, hyphae, blastospores as well as on termite cadavers after injection of blastospores directly in the host hemocoel. The competent blastospores are easily prepared and can be frozen for long-term storage (Ying & Feng, 2006), which will be applicable to other blastospore-producing fungi. However, transformation of frozen/thawed blastospores in *M. anisopliae* is still to be tested. This transformation system has also been applied for the integration of foreign genes into other entomopathogenic fungus such as, *Beauveria bassiana* (Ying & Feng, 2006). In addition, blastospore-based transformation system could be highly preferred for the transformation of the clustered regularly interspaced short palindromic re-peat/associated protein system (CRISPR/Cas9) into fungal cells. A recent study has suggested that the efficiency of this transformation system for CRISPR delivery to *B. bassiana* is high (Chen et al., 2017). Therefore, by combining the use of blastospore-based transformation system and herbicides resistance to GFS and CME, it will be feasible to develop CRISPR/Cas9 as powerful tool for high-efficiency targeted gene knock-out and/or knock-in in *M. anisopliae*, and thus advance understanding of its pathogenesis. The generation of these stable MA-0001 and MA-0002 strains allow the monitoring of the internal infection process within termite hosts. Together, the construction of *gfp*-labelled and over-expressing and knock-out of virulence factors/toxins *M. anisopliae* strains will improve understanding of its pathogenicity to insects and how these factors may influence host immunity including the individual and social levels.

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APPENDIX 1: TERMITE RESPONSE TO DEAD BLASTOSPORES ON THE CUTICLE

As part of Chapter II, we conducted a preliminary behavioural experiment to stablish whether pathogen cues from dead blastospores and a non-pathogenic bacteria trigger elimination response within termite colonies. The aim was to determine whether cannibalism is triggered when termites are cuticular inoculated with dead blastospores, and non-pathogenic bacteria (*Escherichia coli* k-12) as compared to conidia infection.

Eighteen experimental *R. flavipes* colonies were built inside Petri dishes as described elsewhere (Davis et al., 2018). To establish experimental colonies, we introduced a total of 49 healthy termites (not including the focal individual): 46 medium-to-large workers (3-5 mm body length), 2 representatives of the reproductive caste and 1 soldier. Sealed experimental nests were maintain in a dark room at 27°C and 70% humidity for 15 days.

To determine whether cuticular exposure of dead blastospores and non-pathogenic bacteria triggers cannibalism at different incubation time points, 9 focal *R. flavipes* workers (stained with Nile blue) from each colony (11+13 and 5) were anaesthetized with CO_2, then directly exposed to 41,4 nL of a suspension of either dead blastospores (1×10^7 dead blastospores/mL) of *M. anisopliae* (Blastospores-), non-pathogenic bacteria (1×10^4 *E. coli*/mL) or Ringer solution using the Nanoject II. Dead blastospores were generated as described in Chapter II. One bead of *E. coli* from the -70 °C was inoculated in about 50 mL of Luria Bertani medium (LB). The culture was incubated overnight at 150 rpm and 37 °C for 12 hours. 150 µL of the overnight culture of *E. coli* was mixed in 20 mL fresh LB medium and incubated at 37 °C and 150 rpm. Bacteria were grown to exponential phase standardized by optical density at 600 nm. Bacterial suspension was obtained by diluting bacteria in Ringer´s ¼ solution (1×10^4 cells/mL).

Cuticular exposed termites were kept individually in small Petri dishes (35 mm), each containing a Pall cellulose pad moistened with 1 mL of distilled water and then incubated in darkness for 15 hours at 27°C and 70% humidity. After 15 hours of incubation, exposed focal termites were added individually to the Petri dish nests and sealed them with parafilm. After introduction into the Petri dish nests, focal termites were checked to be cannibalized every 3 hours for 12 hours and then at 24 and 48 hours (Table 1).

To test the effect of direct pathogen cues from dead blastospore and non-pathogenic bacteria necessary to induce elimination behaviours, focal termites were exposed either with a suspension containing blastospores killed by autoclaving (dead blastospores), a bacterial suspension or Ringer‘s solution. After 12 hours of introduction into the Petri dish nests, we observed only case of cannibalism within the Ringer treatment. One possible reason for this, could be injury of the termite with the glass needle. Furthermore, we reported only one case of cannibalism in the dead blastospores treatment after 48 hours of introduction. Therefore,

the presence of inactivated or different pathogenic cues indicated that cannibalism is not induce by their external/cuticular presence, but other factors are involved as observed after termites were exposed with active conidia of *M. anisopliae* and a lethal infection was caused (Rosengaus and Traniello, 2001; Davis et al., 2018). We concluded that these cuticular pathogenic cues are not enough to trigger cannibalism within termite colonies.

T**able 1.** Cannibalism of exposed focal termites after introduction into the Petri dish nests.

Treatment	Colony 11+13	Colony 5	Time after introduction
Ringer	–	–	3 h
Blastospores-	–	–	
E. coli K-12	–	–	
Ringer	–	–	6 h
Blastospores-	–	–	
E. coli K-12	–	–	
Ringer	–	–	9 h
Blastospores-	–	–	
E. coli K-12	–	–	
Ringer	–	Petri dish nest 8 (cannibalized)	12 h
Blastospores-	–	–	
E. coli K-12	–	–	
Ringer	–	–	24 h
Blastospores-	–	–	
E. coli K-12	–	–	
Ringer	–	–	48 h
Blastospores-	Petri dish nest 15 (cannibalized)	–	
E. coli K-12	–	–	

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