SOCIAL IMMUNITY IN A NASUTE TERMITE: THE IMPORTANCE OF SOLDIERS

por

LARISSA FREITAS FERREIRA

(Sob Orientação do Professor Paulo Fellipe Cristaldo UFRPE)

ABSTRACT

Eusocial insects have developed collective social immunity mechanisms to reduce the risk of disease within the colony. In addition, the division of labor is closely linked to this immunity, allowing tasks such as foraging and defense to be carried out efficiently, with individuals divided into castes with specific tasks. Among these castes, soldiers play an important role in defense, resource selection and foraging. These defenses involve a mechanical and chemical apparatus, making soldiers a key focus for understanding immunity. Soldiers of Nasutitermitinae colonies have a frontal gland that releases compounds important for alarm communication and defense recruitment. Although it is known that some termites secrete substances that can control microorganisms, the immunological and behavioral responses of termites to the pathogen have not been fully understood. The aim of this thesis was to analyze the role of soldiers and the synergistic effects of their interactions with workers on social immunity in *Nasutitermes corniger* (Motschulsky) (Termitidae: Nasutitermitinae). Our results indicate that soldiers are prominent for their potential to initiate alarm signals in response to fungal threats, and that secretions from the soldier's frontal gland may have an inhibitory effect on fungal growth, thereby enhancing colony immunity. This work provides important insights into how complex social systems can combat diseases.

KEY WORDS: behavioral ecology, insect-pathogen interaction, *Nasutitermes corniger*, *Metarhizium anisopliae*.

IMUNIDADE SOCIAL EM UM CUPIM NASUTO: A IMPORTÂNCIA DOS SOLDADOS

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RESUMO

Os insetos eussociais desenvolveram mecanismos coletivos de imunidade social para reduzir o risco de doenças dentro da colônia. Além disso, a divisão de trabalho está intimamente relacionada a esta imunidade, permitindo que as tarefas como forrageamento e a defesa sejam realizadas de forma eficiente, sendo que os indivíduos são divididos em castas com tarefas específicas. Entre estas castas, os soldados desempenham um importante papel na defesa, seleção de recursos e no forrageamento. Estas defesas envolvem um aparato mecânico e químico, tornando então, os soldados foco para a compreensão da imunidade. Os soldados de colônias de Nasutitermitinae possuem uma glândula frontal que libera compostos importantes na comunicação de alarme e no recrutamento de defesa. Embora saiba que alguns cupins secretam substâncias que podem controlar microorganismos, ainda não foi totalmente compreendido como as respostas imunológicas e comportamentais dos cupins atuam frente ao patógeno. Asssim, o objetivo dessa tese foi analisar o papel dos soldados e os efeitos sinérgicos de suas interações com operários na imunidade social em *Nasutitermes corniger* (Motschulsky) (Termitidae: Nasutitermitinae). Nossos resultados indicam os soldados destacaram-se pelo seu potencial em iniciar sinais de alarme em resposta à ameaças ao fungo, e que as secreções da glândula frontal do soldado pode ter efeito inibitório sobre o crescimento do fungo, aumentando a imunidade da colônia. Assim, este trabalho fornece informações importantes sobre como os sistemas sociais complexos podem combater as doenças.

PALAVRAS-CHAVE: ecologia comportamental, interação inseto-patôgeno, *Nasutitermes corniger*, *Metarhizium anisopliae*.

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Tese apresentada ao Programa de Pós-Graduação em Entomologia, da Universidade Federal Rural de Pernambuco, como parte dos requisitos para obtenção do grau de Doutor em Entomologia.

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por

LARISSA FREITAS FERREIRA

Comitê de Orientação:

Paulo Fellipe Cristaldo – UFRPE

Camila Costa Moreira – Koppert Brasil

José Dijair Antonino S. Júnior – UFRPE

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LARISSA FREITAS FERREIRA

Banca Examinadora:
Paulo Fellipe Cristaldo – UFRPE
Laura Carolina Leal de Sousa – UNIFESP
Flávia Maria da Silva Moura – UFCG
Iago Bueno da Silva – USP
José Dijair Antonino S. Júnior – UFRPE

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Larissa Freitas Ferreira
Doutor em Entomologia

Prof. Paulo F. Cristaldo – UFRPE
Orientador

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SUMÁRIO

Página

CAPÍTULOS

CHAPTER 1

GENERAL INTRODUCTION

Eusociality is rare in the animal kingdom, however, eusocial insects (termites, ants, bees, and wasps) have achieved tremendous ecological success and dominate many terrestrial environments (Wilson, 1971). Eusocial insects generally live in very sophisticated colonies, which are similar to human societies in many ways; they live in densely populated colonies, perform tasks through division of labor, build complex nesting systems, and engage in sophisticated social communication (Sun *et al.* 2018).

Pathogens pose a significant threat to eusocial animals. Insect societies provide ideal conditions for the dissemination of pathogens, because a large number of closely related individuals with potentially very similar immune defenses live together in intimate contact and under homogeneous, environmentally buffered conditions. In addition, low genetic variance has been shown to reduce the chances of successfully resisting several fungus infections, mainly in honeybees and ants (Wilson 1971, Hölldobler & Wilson 2009). According to Schmid-Hempel (2021), such characteristics observed in social groups facilitate the disease transmission, a concept called as "*generalized transmission distance*". To combat pathogen risks, social insects evolved a range of behavioral and physiological strategies to inhibit the spread of diseases, also known as '*social immunity*' (Cremer *et al.* 2007).

Important organisms for studying social immunity in insects are fungal pathogens once they can produce many infectious spores (Rosengaus *et al.* 1998, Myles *et al.* 2002, Cremer *et al.* 2007). After adhering to the cuticle of insects, they enter the body cavity, damage cells, and kill the host (Zhang *et al.* 2024). In contrast, to reduce disease and improve survival, insects have sophisticated multi-defense mechanisms at individual and group levels were favored over evolutionary due to their effects in reducing disease effects and improving insects. For example, termites avoid contact with pathogens by performing vibratory signals to alert nestmates (Rosengaus *et al.* 1999, Bulmer *et al.* 2019). In addition, they can transfer antimicrobial substances through trophallaxis, and disposal of dead bodies, as well as the perform cleaning behavior (*i.e.*, grooming) to remove pathogenic spores from other individuals (Rosengaus *et al.* 1998, Hamilton *et al.* 2011, Rosengaus *et al.* 2011, Sun & Zhou 2013).

Another aspect related to social immunity is the division of labor among non-reproductive individuals. This organization individuals are assigned to specific tasks within the colony, so that the reproductive caste can be protected from external threats. And other castes (*i.e.*, workers and soldiers) perform tasks that may increase the risk of exposure to the pathogen, such as foraging, defense, or disposal of corpses (Noirot 1969, Robinson 2009, Sun & Zhou 2013, Stroeymeyt *et al.* 2014). These castes have several behavioral adaptations associated with specific tasks. Termite soldiers, on the other hand, have lost their ability to feed themselves and are fed by the workers through trophallaxis (Grassé 1984). This behavior is an interesting example of colony division of labor and specialization. The soldier primarily handles the defense task, specializing in this role morphologically, as well as, physiological and neural modifications that control soldier defensive behaviors (Ishikawa *et al.* 2016, Miura & Maekawa 2020). While also contributing to other functions, they can also contribute to the search for food by locating and detecting changes in the availability of food resources (Traniello 1981, Almeida *et al.* 2016, Sacramento *et al.* 2018).

Rhinotermitidae, Serritermitidae, and Termitidae soldiers have developed the frontal gland that produces defensive substances, and a diversity of chemical compounds. These compounds can be irritants, repellents, or pheromones involved in the differentiation of soldiers (Costa-Leonardo & De Salvo 1987, Costa-Leonardo *et al.* 2023). In the genus *Nasutitermes* (Termitidae: Nasutitermitinae), soldiers have an ampoule-shaped head that houses the frontal gland. This gland secretes chemicals mainly terpenes, which may also show fungicidal activity (Rosengaus *et al.* 2000, Fuller 2007).

The soldiers of *Nasutitermes costalis* (Holmgren) can control the growth of a nest microbe by releasing terpenoid secretions from their frontal gland (Rosengaus *et al.* 2000). Therefore, termites have developed the ability to produce several antimicrobial substances. These include α-pinene and limonene in *Nasutitermes* species, trinervitan and n-hexanoic acid in *Zootermopsis* species (Rosengaus *et al.* 2004, Zhao *et al.* 2004), and naphthalene found in the inner nest wall of *Coptotermes formosanus* Shiraki (Chen *et al.* 1998). The frontal gland of the soldier therefore appears to have several functions in addition to defense, such as fungicidal activity throughout the colony (Rosengaus *et al.* 2000, Fuller 2007).

The ability to produce antimicrobial substances is essential for termite survival. Antifungal secretions, together with behavioral defenses, may contribute to colony immunity. It is well known that soldiers have a recognized role in defense and foraging. However, their contribution to other functions such as colony immunity, particularly against pathogen threats, has been less understood. Understanding the defense mechanisms of termites is also crucial. It has significant implications for the immunology, behavior and ecology of this group. Therefore, the aim of this thesis aimed to understand how the social organization of termites (*i.e.,* castes) contributes to social immunity, in addition to exploring the defensive, behavioral, and physiological strategies capable of modifying the fungal infection process.

Termite immunity

The individual immune system of insects comprises three main immune pathways, immune deficiency (IMD), Toll and Janus kinase-signal transducer, and activator of transcription (Rolff & Reynolds 2009). In addition, termites have evolved social immune traits at the group level to prevent the spread of infectious diseases within colonies (Cremer *et al.* 2019). The immune system of termites comprises both the individual innate immunity and behavioral defenses at the group level (Cremer 2019).

Fungal-termite infection occurs in several stages. Initially, the fungus adheres to the host cuticle; it is at this stage that the entomopathogenic fungi produce volatile organic compounds (VOCs), which can induce behavioral or physiological responses in the host (Baverstock *et al.* 2009). For example, the termite *Macrotermes michaelseni* (Termitidae: Macrotermitinae) can distinguish between virulent and avirulent strains of *M. anisopliae* and *B. bassiana* based on VOC profiles (Mburu *et al.* 2009). In *Coptotermes formosanus* Shiraki (Rhinotermitidae), fungal odor promotes mutual grooming and attack of the contaminated nestmates (Yanagawa *et al.* 2011). Posteriorly, the fungus comes into contact and fixed with the host cuticle; at this point, it might cause the synthesis of antimicrobial compounds in the cuticle or stimulate the cleaning of nestmates (*i.e.,* grooming) (Roy *et al.* 2006). Then, the fungi enter the hemocoel and other tissues; insect cellular and humoral immune responses are activated, following by changes in the behavioral responses, potentially leading to host death (Zhang *et al.* 2024).

Pathogens may cause behavioral changes in both infected and uninfected from the same colony individuals. Some termite species, for instance, exhibit hygienic behaviors (*e.g.,* grooming), active removing or treating the corpses of individuals that died from fungal infection, as well as trophallaxis, vibratory behavior (*i.e.,* alarm reaction), and antennation (Rosengaus *et al.* 2011, Sun & Zhou 2013, Liu *et al.* 2019, Ferreira *et al.* 2023). Furthermore, termites can incorporate chemical/antimicrobial compounds into nest material to prevent entomopathogens from establishing themselves in their environment. Naphthalene, an antiseptic and anthelmintic, is found in the nest wall of *C. formosanus* (Chen *et al.* 1998). Similarly, termite salivary glands release two chemicals with antifungal activity: termicin in Reticulitermes termites (Hamilton & Bulmer 2012) and Gram-negative binding protein 2 (GNBP2) in *N. corniger* and *Mastotermes darwiniensis (*Bulmer *et al.* 2009, He *et al.* 2018). Furthermore, termites produce compounds with antimicrobial activity (Rosengaus *et al*. 2000, Fuller 2007, Otti *et al.* 2014, Mitaka *et al.* 2017). The immunological responses triggered by termites can result in the production of antimicrobial enzymes and peptides, including spinigerin and termycin in *Pseudacanthotermes spiniger* (Termitidae: Macrotermotinae) (Lamberty *et al.* 2001), termycin and tGNBP-2 in *N. corniger* (Bulmer *et al.* 2009), and lysozyme in *Reticulitermes speratus* (Matsuura *et al.* 2007).

System studied: termites and entomopathogenic fungi

The nasute termite *N. corniger* is a Neotropical species with a wide distribution from Mexico to Argentina (Scheffrahn *et al.* 2005a, Santos *et al.* 2017). The species *Nasutitermes polygynus* from New Guinea and *Nasutitermes costalis* from the Caribbean Islands have been synonymized with *N. corniger*, a species native to Central and South America (Scheffrahn *et al.* 2005a,b). Individuals of *N. corniger* can produce enzymes that break down cellulose, allowing this group to feed on a wide range of food sources (Albuquerque *et. al.* 2014). In addition, this species has a network of nests connected by tunnels (called '*polycalic nests*'), which allows the royal couple to move quickly if contaminated (Costa-Leonardo 2002, Fontes & Milano 2002). Because of its feeding habits, it is considered a pest in agricultural and urban environments (Constantino 2002, Scheffrahn *et al.* 2014).

The entomopathogenic fungus *Metarhizium anisopliae* (Metschn.) Sorokin infects a range of hosts within of Insecta group. Stages of adhesion, germination, penetration, invasion, colonization and dissemination characterize its life cycle (Neves & Alves 2004). The choice of *M. anisopliae* is due to this fungus species showed greater virulence in previous studies (Chouvenc *et al.* 2009, Ambele 2020, Singha *et al.* 2011). In addition, it is known that this fungus is naturally associated with termites (Milner *et al.* 1998).

The thesis was divided into four chapters. The Chapter 1 is the general introduction, that provides the reader an overview of the issue and the general objective of the study. Chapter 2 consists of the article "**Caste composition contributes to improve the social immunity in a nasute termite species exposed to entomopathogenic fungus**" published in the journal *Ecological Entomology,* showing the role of the two castes (*i.e.,* workers and soldiers) in the mechanisms of social immunity in *Nasutitermes corniger* (Motschulsky). The Chapter 3 presents the article **"Secretions of the frontal gland of a nasute termite soldier: the role in the defense against an entomopathogenic fungi"**, which analyzes the effect of the frontal gland on fungal infection in groups of *N. corniger*. The Chapter 4 summarizes the final considerations of this thesis.

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CHAPTER 2

CASTE COMPOSITION CONTRIBUTES TO IMPROVE THE SOCIAL IMMUNITY IN NASUTE TERMITE SPECIES EXPOSED TO ENTOMOPATHOGENIC FUNGUS

LARISSA FREITAS FERREIRA[1,2], CÁTILA R. SILVA[2], RENAN E. C. SANTOS[2], CAMILA C. MOREIRA[3], PAULO FELLIPE. CRISTALDO[1,2]

[1] Post Graduate Program in Entomology, Department of Agronomy, Universidade Federal Rural de Pernambuco, Recife, Brazil

[2] Laboratory of Insect Ecology, Department of Agronomy, Universidade Federal Rural de Pernambuco, Recife, Brazil

[3] Koppert Biological Systems, Piracicaba, Brazil

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ABSTRACT – The evolution of a reproductively altruistic caste contributed to the success of eusociality through the division of labour. In termites, the proportion of soldiers in the colonies increased throughout the group's evolution. In Nasutitermitinae, soldiers have a crucial role in defense, resource selection and foraging. However, the role of soldiers and workers in colony immunity is still poorly understood. 2. Here, we evaluated the role of mixed caste groups in the social immunity of *Nasutitermes corniger* (Termitidae: Nasutitermitinae) exposed to the entomopathogenic fungi *Metarhizium anisopliae* (Metsch.). We tested the hypothesis that the presence of workers and soldiers optimizes defense against pathogens, and this defense is increased when the soldier ratio is closer to what naturally occurs in colonies. Our results showed that mixed caste groups of *N. corniger* exposed to *M. anisopliae* survive longer compared to groups with only one caste. In addition, termite groups that died from fungi were less likely to show fungal infection in mixed caste groups. The behavioural observations suggest that in the natural proportion of soldiers, allogrooming and trophallaxis play a crucial role in the control of disease and its death hazard in termites. Soldiers may play an important role in colony immunity, being able to start the alarm signal indicating the threat of pathogens. Our results show that the presence of the two castes promotes possible mechanisms for socially mediated immunity. This study may help in the understanding of the function of the soldiers and workers and the significance of pathogens in termite eusocial evolution.

KEYWORDS: behaviour, *Metarhizium anisopliae*, *Nasutitermes corniger*, survivorship

A COMPOSIÇÃO DE CASTAS CONTRIBUI PARA MELHORAR A IMUNIDADE SOCIAL EM UMA ESPÉCIE DE CUPIM NASUTO EXPOSTA À UM FUNGO ENTOMOPATOGÊNICO

RESUMO – A evolução de uma casta reprodutivamente altruísta contribuiu para o sucesso da eussocialidade através da divisão do trabalho. Nos cupins, a proporção de soldados nas colônias aumentou ao longo da evolução do grupo. Em Nasutitermitinae, os soldados têm um papel crucial na defesa, seleção de recursos e forrageamento. No entanto, o papel dos soldados e operários na imunidade das colónias de cupins ainda é pouco compreendido. No presente trabalho, avaliamos o papel de grupos de castas mistas na imunidade social de *Nasutitermes corniger* (Termitidae: Nasutitermitinae) exposto ao fungo entomopatogênico *Metarhizium anisopliae* (Metsch.). Testamos a hipótese de que a presença de operários e soldados otimiza a defesa contra patógeno, e essa defesa é aumentada quando a proporção de soldados está mais próxima do que ocorre naturalmente nas colônias. Nossos resultados mostraram que grupos de castas mistas de *N. corniger* expostos a *M. anisopliae* sobrevivem por mais tempo em comparação com grupos com apenas uma casta. Além disso, os grupos de cupins que morreram de fungos tiveram menor probabilidade de apresentar infecção fúngica em grupos de castas mistas. As observações comportamentais sugerem que, na proporção natural de soldados, o *allogrooming* e a trofalaxia desempenham um papel crucial no controlo de doenças e no seu risco de morte em cupins. Os soldados desempenham um papel importante na imunidade das colônias, sendo capazes de iniciar o sinal de alarme indicando a ameaça de patógeno. Nossos resultados mostram que a presença das duas castas promove possíveis mecanismos de imunidade mediada socialmente. Este estudo pode ajudar na compreensão da função dos soldados e operários e do significado dos patógenos na evolução eussocial dos cupins.

PALAVRAS-CHAVE: comportamento, *Metarhizium anisopliae*, *Nasutitermes corniger*, sobrevivência

Introduction

Insects that live in eusocial groups can have many benefits of the social lifestyle, such as increased ability to find and collect food resources as well as increased defense against competitors and/or predators (Nowak *et al.* 2010). On the other hand, living in societies is not always beneficial: the high probability of exposure to pathogens is one cost associated with this behaviour (Schmid-Hempel 1998, Nowak *et al.* 2010, Chouvenc *et al.* 2021). Indeed, parasites and pathogens are reported as selective forces acting on the evolution of eusociality (Rosengaus & Traniello 1993). Eusocial insects live in densely populated colonies, therefore, encounters among individuals are frequent. In addition, nests are usually microbial-rich environments. Thus, the transmission of pathogens among individuals may be higher compared to solitary insects (Rosengaus *et al.* 2011).

A range of collective mechanisms (so-called '*social immunity*') has evolved in eusocial insects, ensuring to reduce the sanitary risks, and also complementing the physiological mechanisms at the level of the individuals (*i.e.,* individual immunity) (Chouvenc & Su 2012, Rosengaus *et al.* 2011). Among these collective mechanisms, prophylactic behaviors (*i.e.*, mutual grooming, proctodeal and oral trophallaxis and nest architecture) result in efficient individual and social immunity (Liu *et al.* 2019, Rosengaus *et al.* 2011).

Over the last decades, a series of studies have been conducted to understand the mechanisms involved in the collective strategies of resistance to pathogens in insect societies (Cremer *et al.* 2017, Schmid-Hempel 2017). Ants, for instance, use their glandular secretion and cultured bacteria in the colonies to prevent microbial growth (Malagocka *et al.* 2019). In termites, a series of studies have shown mechanisms to prevent the spread of infections in the colonies of phylogenetic basal species.

Rosengaus *et al.* (1999), for example, demonstrated that individuals of *Zootermopsis angusticollis* (Isoptera: Archotermopsidae) elicit an alarming behaviour in the presence of fungal conidia. The secretions present in the sternal gland of *Z. angusticollis* are responsible for inhibiting the growth of the entomopathogenic fungus *Metarhizium anisopliae* (Rosengaus *et al.* 2004).

Although phylogenetically derived termites account for approximately 85% of all termite species, fewer studies have been conducted to examine the mechanisms to prevent the spread of pathogens in their colonies. Bulmer *et al.* (2009) have shown that *Nasutitermes corniger* (Termitidae: Nasutitermitinae) incorporates β,1-3 glucanase in their nest, which cleaves and releases components, such as glucans, to avoid pathogens. The terpenoids present in the frontal gland of soldiers of the *Nasutitermes* species are known to inhibit the germination *of M. anisopliae* (Rosengaus *et al.* 2000).

Throughout the evolution of the termite group—from basal to derived species there is a trend to increase (1) the complexity of soldiers' defensive arsenal (Cristaldo *et al.* 2015; Šobotník *et al.* 2010) and (2) the proportion of soldiers inside colonies (Noirot & Darlington 2000). The soldier caste has specialized morphologies for defense against competitors, natural enemies (predators, parasitoids and pathogens) and intruders (*i.e.,* a non-nestmate conspecific, or a different termite species) (Noirot & Darlington 2000). However, in the few last years, studies have shown that soldiers also present a crucial role during resource selection and foraging (Traniello 1981, Casarin *et al.* 2008, Almeida *et al.* 2016, Sacramento *et al.* 2020). In species from Nasutitermitinae subfamily, soldiers present the frontal gland on their head, which synthesizes a terpenoid secretion from a projection called '*nasus*', which functions as alarm communication to defensive recruitment (Prestwich 1984). Soldiers in these species can represent up to 30% of all individuals (Haverty 1977). *N. corniger* build the nest above the soil surface (*i.e.*, arboreal nest), with a network of galleries to connect the nest with food sources (Abe 1987). It has been hypothesized that the behaviour of termites to build their nest in trees was a response to the selective pressure of pathogens. This species, however, is still exposed to infection risk from fungal propagules in the forest soil via the nest substrate and foraging galleries.

Despite the mechanisms involved in the defense against pathogens the question of how the composition of castes in termites can contribute to colony-level immunity, as well as behaviors that can improve chances of survival, is still poorly understood. Here, we evaluated the role of workers and soldiers in the social immunity of *N. corniger* groups exposed to the entomopathogenic fungi *M. anisopliae*. Specifically, we tested the hypothesis that social immunity is enhanced in the presence of both castes and that such a pattern can be explained by a greater number of prophylactic behaviors in groups with a natural proportion of soldiers in the colony (*i.e.*, 30% of soldiers).

Materials and Methods

Ethics statement. Permits for termite collection were issued by ICMBio—IBAMA to PFC (#47652). No specific permits were required for the described studies undertaken in the laboratory with a non-endangered or protected species.

Termite colonies. Arboreal nests of *N. corniger* were collected at the campus ‘SEDE’ of the Rural Federal University of Pernambuco (UFRPE) (8º04’03’’S, 34º55’00’’W), in the municipality of Recife, state of Pernambuco, Brazil. According to Köppen classification, the climate in the area is characterized as ‘subtropical humid or sub-humid' ('Aw'), with an average annual temperature of 25.7°C and an average annual precipitation of 988 mm (Climate 2022).

Nests with active colonies of *N. corniger* were removed from the trees and taken to the Laboratory of Insect Ecology at UFRPE to be used for the bioassays. In the laboratory, colonies were placed separately in plastic boxes with moistened cotton. Colonies were kept in the laboratory after being taken from the field for 24 h before the bioassays. A total of five colonies were sampled to conduct bioassays of survivorship and behaviour. All bioassays were performed under controlled conditions (25 ± 2 ºC, 70 ± 2% U.R., and low light conditions).

Conidia preparation. We selected the species *M. anisopliae* (strain ESALQ-E9) because this fungus species showed greater virulence in previous tests (see Appendix S1). In addition, studies of virulence and defense mechanisms in termites were performed with *M. anisopliae* as a model entomopathogen (Chouvenc *et al.* 2009, 2011, Rosengaus *et al.* 2011).

The entomopathogenic fungus *M. anisopliae* (strain ESALQ-E9) was obtained from the collection of Entomopathogenic Microorganisms 'Prof. Sérgio Batista Alves' of the Laboratory of Pathology and Microbial control (ESALQ/University of São Paulo, Brazil) and streaked onto potato dextrose agar (PDA) plates supplemented with streptomycin, to prevent bacteria contamination. All inoculated plates were incubated for 7 days in a climate chamber at 26 °C under a 12 L:12D light cycle. The conidia suspension was prepared by scraping the conidia from plates using a flamed metallic loop. Conidia were then transferred to a 0.05% Tween 80 solution.

The conidia suspension was serially diluted and vortexed for 5 min. For conidia counting, 200 μL from the diluted suspension at 10^{-3} was placed in an improved Neubauer haemocytometer. The conidia spores were counted under a Leica compound microscope at 40× magnification. After the number of conidia in the stock was determined, conidial suspensions were prepared in Tween 80 solution at 0.05% and the concentration was adjusted to 1.0×10^7 conidia/mL.

Experimental procedure. Survival and behaviour of *N. corniger* were evaluated by manipulating

caste composition in the group (workers: soldiers), and the fungal exposure. To do so, four termite groups with 10 individuals were established with a different number of workers and soldiers. The following treatments were established: (1) 10 workers and zero soldiers (0% soldiers; 10 W: 0S), (2) seven workers and three soldiers (30% soldiers; 7 W: 3S), (3) five workers and five soldiers (50% soldiers; 5 W: 5S) and (4) zero workers and 10 soldiers (100% soldiers; 0 W: 10S). The choice of 10 individuals of termites in the arenas was a compromise with optimal density to maximize inter-individual contact (Miramontes & DeSouza 1996).

The bioassays were conducted in Petri dishes (80 × 15 mm), whose internal bottom surface was lined with filter paper. Only healthy and non-injured individuals were used. Termite groups were treated with 1.0×10^7 conidia/ml of *M. anisopliae* suspended in 0.05% Tween 80 or a 0.05% Tween 80 solution (control). The fungal suspension (2 mL) was sprayed on each plate individually at a distance of 30 cm to reach all termite bodies. Control replicates were consistently

set up before the spore-exposure replicates to avoid contamination of controls with conidia. The Petri dishes were maintained in a climate chamber at 26 °C under a 12L:12D light cycle and were observed daily for 8 days. The termite mortality was determined by a lack of a movement reaction.

Cadavers were removed daily, and the surface was sterilized with 95% alcohol (Gao *et al.* 2012). The cadavers were placed in a wet chamber to confirm mortality by entomopathogenic fungi. Each cadaver was observed to confirm if the fungus emerging from the insect body matches the applied fungus. The presence of the fungus was confirmed by the visual observation of the withe hyphae and green masses of conidia (Humber 2012). The Petri dishes were incubated at 26 °C under a 12L:12D light cycle until the growth of visible mycelia and the production of conidia on termite carcasses. We estimated the prevalence of death from infection caused by *M. anisopliae* of fungi varying from 0.0 to 1.0 by dividing the number of termites in the group with fungi by the total number of termites. Three repetitions per group composition were performed for each of the five colonies tested, totalling 120 bioassays (with fungi and without fungi [control]).

After 5 min of treatment and control applications, we conducted behavioural observation bioassays. These bioassays consisted of video recording, for posterior analysis, the reaction of termites over 2 minutes under normal white light. The recording was made using a digital camera. All videos were processed using blinded methods to minimize observer bias. The following behaviors were counted for all individuals in the Petri dish during each observation: antennation,

trophallaxis, grooming, vibratory movements, evasion, fighting and biting. The vibratory movements consisted of longitudinal movements along the anterior-posterior axis of the termite body. Three repetitions per group composition were performed for each of the five colonies tested, totalling 120 bioassays (with fungi and without fungi [control]).

Data analyses. The statistical analyses were carried out in R statistical software (R Development Core Team, 2019) using generalized linear mixed models (GLMMs), followed by residual analysis to check the suitability of the models. Model simplification, when necessary, was conducted by removing non-significant explanatory variables from the initial model and evaluating subsequent changes in the deviance. In all analyses, the average from the three repetitions per colony was used as true repetition.

To check whether the presence of the entomopathogenic fungus ($x\text{-}var_1$), the caste composition in the group ($x\text{-}var_2$) and the interaction of these factors would affect the mean time to death of termites (*y-var*), data were initially submitted to censored survival analysis with Weibull distribution to access the mean time to death (hours) of individuals from each replicate, following the procedure described in DeSouza *et al.* (2009). Then, these data (the mean time to death of termites [*y-var*]) were submitted to ANODEV with Gaussian error distribution. Group composition and fungal exposure were used as a fixed factor, and the colony identity was used as a random factor. To assess the significance of treatment effects, we performed a Wald test. Pairwise comparisons of estimated marginal means ('*EMMeans'*) corrected by the false discovery rate method were used as post hoc tests.

To verify the effect of group composition (*x-var*) in the prevalence of *M. anisopliae* among dead termites (*y-var*), data were submitted to ANODEV with Binomial error distribution. Group composition was used as a fixed factor, and the colony identity was used as a random factor. To assess the significance of the treatment effect, we performed a Wald test. Pairwise comparisons of estimated marginal means ('*EMMeans'*) corrected by the false discovery rate method were used as post hoc tests.

To check whether the caste composition (*x-var*$_1$) and the presence of the entomopathogenic fungus (*x-var*$_2$) would affect the termite behaviors, data were submitted to ANODEV with Poisson error distribution. Models were validated by inspecting residuals and testing for overdispersion. Group composition and fungal exposure (with or without fungi [control]) were used as a fixed factor, and the colony identity was used as a random factor. To assess the significance of the treatment effects, we performed a Wald test. Pairwise comparisons of estimated marginal means (‘*EMMeans*’) corrected by the false discovery rate method were used as post hoc tests.

To test the relationship among behaviors in each group composition in the presence or absence of the fungus (control), data were submitted to Pearson’s correlation analysis to determine which behaviors were correlated.

Results

In the presence of fungus, mixed caste groups have higher survival and low fungus prevalence. In general, *N. corniger* groups exposed to *M. anisopliae* survived 66.71 ± 5.20 h while groups without *M. anisopliae* survived 91.55 ± 3.71 h. There was a significant effect of the interaction between fungal exposure and group composition on the mean time to death of termites (GLMM; Table 1). The post hoc test showed that mixed caste groups containing 30% and 50% of soldiers (*i.e.,* 7W:3S and 5W:5S) do not differ from each other in groups exposed to fungus (*EMmeans*; Table 2). However, single caste groups with fungus significantly differ in the mean time to death (*EMmeans*; Table 2); groups composed only of workers survive longer than those with only soldiers (Figure 1). In the control group, however, there was no significant effect of caste composition on the mean time to death of termite groups (*EMmeans*, Table 2). In general, the control group survives a similar mean time to those in the mixed caste groups with fungus (Figure 1). Survival curves showing the dynamic of death in groups with and without fungi in different caste compositions are shown in Figure 2.

For groups of termites exposed to fungus, the confirmation rate for *M. anisopliae* infection was high. The effect of the presence of the two castes on the prevalence of fungus was significant (GLMM; Chisq = 3.68, *d.f.* = 20, p = 0.04). The termite groups with 30% of soldiers (7 W:3S) showed a lower prevalence of fungus in dead termites followed by groups with 50% of soldiers (5W:5S) (Figure 3). No significant differences were observed in groups with 0% and 100% of soldiers (10W:0S and 0W:10S, respectively) (Figure 3). The 70%–100% confirmation of infection by *M. anisopliae* reflects the susceptibility of isolated individuals to fungal infection and not mortality caused by some other factor. No cadaver testing positive for fungal infection was observed in the control groups (untreated).

Effect of fungus and caste composition on the behaviour of termite group. We observed behavioural interactions following exposure to fungal spores that caused an increase in the relative frequency of behaviors. In general, antennation, trophallaxis, grooming and vibratory movements were significantly affected by caste composition in the group and the presence of the fungus (see Table 3; Figure 4). Evasion, fighting and biting were neither exhibited by termites in

treatment nor control groups. All observed behaviors were higher in groups exposed to fungus compared to non-exposed groups (control).

In general, groups with fungus present a higher number of antennation in single caste group containing only soldiers followed by single caste group with only workers and mixed caste groups, which do not differ among them (Figure 4a). The number of grooming and trophallaxis

in termites exposed to the fungus was higher in mixed caste groups containing 30% of soldiers compared to other caste composition groups (Figure 4b, c). The vibratory movements were higher in mixed caste groups and single caste group with soldiers compared to single caste group with workers (Figure 4d).

Pearson's correlation analysis among caste composition groups with and without fungi showed that there was no correlation among the behaviors in the different group compositions in the control (Figure S2). On the other hand, grooming and trophallaxis were significantly correlated ($p < 0.05$) in groups with fungus containing 30% of soldiers ($r = 0.947$) (Figure S3).

Discussion

To avoid potential pathogens, eusocial insects have evolved a robust immune system comprising individual and social levels. However, the effect of caste composition in the response to infection at the social level remains poorly understood in higher termite species. This study explored the role of mixed caste groups in the social immunity of *N. corniger* exposed to the entomopathogenic fungi *M. anisopliae*. In general, our results showed that mixed caste groups moderated susceptibility to infection, as evidenced by the positive effect of the mixed group on mean time to death after exposure to the fungal pathogen and the lower prevalence of fungi in dead termites. In addition, our results showed that *N. corniger* groups with the natural proportion of soldiers (*i.e.*, 30% of soldiers; 7W:3S) increase the prophylactic behaviors and those soldiers seem to play an essential role in the survival benefits of *N. corniger* when infected with *M. anisopliae*.

The effect of mixed caste groups on the colony immunity of termite species has been observed in a few studies. Here, we found that the mixed caste groups of *N. corniger* exposed to fungi survived longer compared to single caste groups (Figures 1 and 2). The higher survival in the mean time to death can be explained by the lower prevalence of *M. anisopliae* in dead termites (Figure 3). Such results together may be related to disease resistance. These results corroborate with was already found in *Nasutitermes exitiosis* (Termitidae: Nasutitermitinae), in which mixed caste groups (soldier and worker) resist infection by *M. anisopliae* better than single caste groups (soldier or worker) (Rath 2000). In fact, Gao *et al.* (2012), showed that defense in termites might depend on the nature of the social interactions as inferred from group composition. Disease risk is lower when there is a division of labour because caste diversity can limit the spread of pathogens
through the transfer of caste-specific physiological defenses or behavioural defense mechanisms (Boomsma *et al.* 2005, Elliot & Hart 2010). The results found in the present study show a type of'*social immunity*' analogous to that found in other social insects (Naug & Gadagkar 1999), including the honeybee *Apis mellifera* (Hymenoptera: Apidae) (Evans *et al.* 2006).

The mixed caste group showed a lower prevalence of *M. anisopliae* in dead termites compared to the single caste group, indicating that soldiers together with workers maximise the resistance to pathogens in groups of *N. corniger*. Zeng *et al.* (2022) report that workers of *Coptotermes formosanus* (Isoptera: Rhinotermitidae) implemented sanitary care, with no caste priority, to the infected soldiers or their infected nestmate workers. Furthermore, workers sacrificed their survivorship to maintain the soldier proportion of the group. Interestingly, the mixed caste group composed of a natural proportion of soldiers (*i.e.*, 30% of soldiers; 7W:3S) showed a lower prevalence of fungus compared to 50% of soldiers (Figure 3). The results from our behavioural bioassays suggest that such results must be related to the increased prophylacticbehaviors in mixed caste groups composed of the natural proportion of soldiers.

In general, behavioural observations revealed that the presence of fungal spores of *M. anisopliae* significantly increased the number of *N. corniger* prophylactic behaviors compared to control. The number of antennation, grooming, trophallaxis and vibratory movements were significantly affected by caste composition in groups exposed to *M. anisopliae* (Figure 4). To protect the colony against fungal infection, termites need to recognise pathogenic conidia on the body surface and remove them. Dhanarjan (1980) suggested that antennal contact with nestmates is essential to begin grooming once this behaviour is initiated through the detection of foreign organisms on their body surface. Therefore, termites also can recognise pathogens by antennal contact (Yanagawa *et al.* 2009).

The removal of conidia via grooming can prevent infection of nestmates. The soldier caste in *N. corniger* relies on grooming by workers because of the missing specialised mandibles. However, even though soldiers cannot clean themselves, they produce volatile substances with an antifungal activity that contributes to the survival of workers (Mitaka *et al.* 2017, He *et al.* 2018, Rosengaus *et al.* 2000). Soldiers of termites have evolved mechanical and chemical defenses. Rosengaus *et al.* (2007) showed that the *Z. angusticollis* soldiers can upregulate antimicrobial proteins that are more effective at reducing fungus viability than antimicrobials expressed by pseudergates (worker-like caste found in some species). He *et al.* (2018) observed that exocrine oral secretions produced by soldiers of *Mastotermes darwiniensis*

(Isoptera: Mastotermitidae) contain a high concentration of proteins involved in immunity and they may protect nestmates against fungal infection.

The trophallaxis, which allows the interindividual transfer of materials, can occur for different purposes, such as nutrition, control of larval growth and development, nestmate recognition, and immune defense (Liu *et al*. 2019). In termites, there is evidence that trophallaxis plays a role in sharing the gut microbiota (Brune & Dietrich 2015, Nalepa 2015). But when exposed to pathogens, termites can benefit from the distribution of non-lethal inoculate dosages or immune proteins through trophallaxis (Rosengaus *et al.* 2011).

A higher number of grooming and trophallaxis (*i.e.*, hygienic behaviour) was observed in mixed caste groups composed of 30% of *N. corniger* soldiers (7 W: 3S); showing that the natural caste ratio of *N. corniger* triggered a lower susceptibility to infection (Figure 4b, c). Pearson's correlation analysis among group composition in termites with and without fungi showed that there was no correlation among the behaviors in the different group compositions in the control (Figure S2). On the other hand, grooming and trophallaxis were significantly correlated ($p < 0.05$) in groups with fungus containing 30% of soldiers ($r = 0.94$) (Figure S3). Interestingly, trophallaxis and grooming are behaviors that can complement each other, oral secretions with protective functions may be passed among nestmates by trophallaxis and spread on the insect cuticle in grooming. For example, pathogen recognition proteins (Gram-negative bacterial-binding proteins—GNBPs) and antimicrobial peptides (termicins) were found in *N. corniger* (Bulmer *et al.* 2009, Bulmer & Crozier 2004). Termites incorporate this protein into nest building material, which can act as a sensor into the nest, cleaving and releasing pathogenic components. It may prepare the termites to improve their defense mechanisms (Bulmer *et al.* 2009).

In addition, Rosengaus *et al.* (2000) showed that the antifungal properties of the frontal gland secretions of nasute termite soldiers, *Nasutitermes costalis* and *Nasutitermes nigriceps* inhibited the germination of *M. anisopliae* conidia.

A higher number of vibratory movements were observed only in groups with soldiers (30%, 50% and 100%) (Figure 4d). This result may indicate that the soldier perceives the pathogen and communicates with the group. In termites, individuals in direct contact with a pathogen exhibit a vibratory behaviour (*i.e.,* alarm reaction), communicating the presence of pathogens to nestmates. In another study, it was observed that soldiers and workers of the *Z. angusticollis* triggered a vibratory alarm behaviour during and after exposure to high concentrations of *M. anisopliae*. However, pathogen alarm behaviour involves antero-posterior and dorso-ventral motions and without apparent sound, differing of the alarm by nest disturbance, that involves producing an audible sound, per alternately hitting the roof and floor of the nest galleries with the head of soldiers (Rosengaus *et al.* 1999).

The caste of the soldiers may represent an essential element of defense against fungi microorganisms. In the absence of workers, soldiers set off the alarm performing high vibratory movements, but they are cleaned by the workers, which must decrease the mean time to death in groups with only soldiers because they cannot clean themselves (see Figure 1). On the other hand, in the absence of soldiers, there is a higher mortality of workers because there must be less detection and less alert, with less cleaning and more death (Figure 1). Therefore, there is a higher prevalence of fungi in dead individuals with 0 and 100% of soldiers (Figure 3), that is, when there is a single caste. These results together with others found in the present study highlight the importance of the two castes (workers and soldiers) in the defense against pathogens.

Studies have shown that several mechanisms are important in determining termite survival in a group, including chemical protection, the nest architecture, the spatial distribution of nestmates in the group size and the rates of social interaction, besides the group composition, which may be also a determinant in survival. These mechanisms require interactions between group members to limit the spread of pathogens at the colony level (*i.e.,* social immunity) and reduce the risk of infection of individuals (*i.e.,* individual immunity). Previous work has demonstrated that *Nasutitermes* soldiers produce biochemical and physiological mechanisms to prevent infection (He *et al.* 2018), but the association of the group composition and proportion of soldiers with pathogens, as well as behavioural changes when fungal pathogens are present and when individuals are infected, has not been described. Therefore, the results obtained in the present study highlight the importance of soldiers and workers of infection control from the colony level. In addition, the results add new perspectives to understand better the initial selection pressures and mechanisms driving the initial emergence of social immunity in species living in a group.

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Table 1. Effect of caste composition, treatment (with and without fungus) and the interaction between caste composition and treatment on mean time to death of *Nasutitermes corniger*.

Source	**Chi-sq**		**P**	
Caste composition (CC)	23.85	3	< 0.001	***
Treatment (T)	29.54	1	< 0.001	***
CC : T	7.83	3	0.04	*

Note: Generalized Linear Mixed Modelling (Analysis of Deviance – Type II Wald $\chi2$ tests) under Gaussian error distribution.

Table 2. Summary of post-hoc test (EMMeans) testing the effect of caste composition, treatment (with and without fungus) and the interaction between caste composition and treatment on the mean time to death of *Nasutitermes corniger* groups.

Caste composition	**trat**	***EMMeans***	**s.e.**	***df***	**confidential interval (ci)**		**group***
					lower	**upper**	
10W:0S	with	58.8	7.46	22,3	36.3	81.4	a
7W:3S	with	79.9	7.46	22,3	57.4	102.5	b
5W:5S	with	84.5	7.46	22,3	62.0	107.0	b
0W:10S	with	43.6	7.46	22,3	21.1	66.1	c
10W:0S	with	80.3	7.46	22,3	57.8	102.8	b
7W:3S	with	94.2	7.46	22,3	71.6	116.7	b
5W:5S	with	101.6	7.46	22,3	79.1	124.1	b
0W:10S	with	90.1	7.46	22,3	67.6	112.6	b

* Group with same letter did not differ statistically (P> 0.05)

Table 3. Effect of caste composition, treatment (with and without fungus) and the interaction between caste composition and treatment on behaviors of *Nasutitermes corniger* groups.

Source	***Chisq***	***d.f***	***P***	
response variable: antennation				
caste composition (CC)	8.57	3	0.03	*
treatment (T)	61.30	1	0.0001	***
CC: T	8.10	3	0.04	*
response variable: grooming				
caste composition (CC)	31.22	3	0.0001	***.
treatment (T)	15.81	1	0.0001	***
CC: T	7.35	3	0.06	*n.s.*
response variable: trophallaxis				
caste composition (CC)	14.59	3	0.002	**
treatment (T)	11.43	1	0.0001	***
CC: T	3.30	3	0.34	*n.s*
response variable: vibratory movements				
caste composition (CC)	9.26	3	0.002	**
treatment (T)	8.07	1	0.004	**
CC: T	1.81	3	0.61	*n.s.*

Note: Generalized Linear Mixed Modelling (Analysis of Deviance—Type II Wald $\chi 2$ tests) under Poisson error distribution. *: P=0.05; **: P<0.01; ***: P < 0.001.

Figure 1. Mean time to death of *Nasutitermes corniger* in different soldier's proportions in the group with and without fungi (control). Boxplots followed by the same letters do not significantly differ by EMMeans post-hoc test. Raw data are represented by blue and yellow points, the mean values by the black points, blue and yellow horizontal lines demarcate the central value of the data set, and vertical lines indicate the minimum and maximum value of the data set.

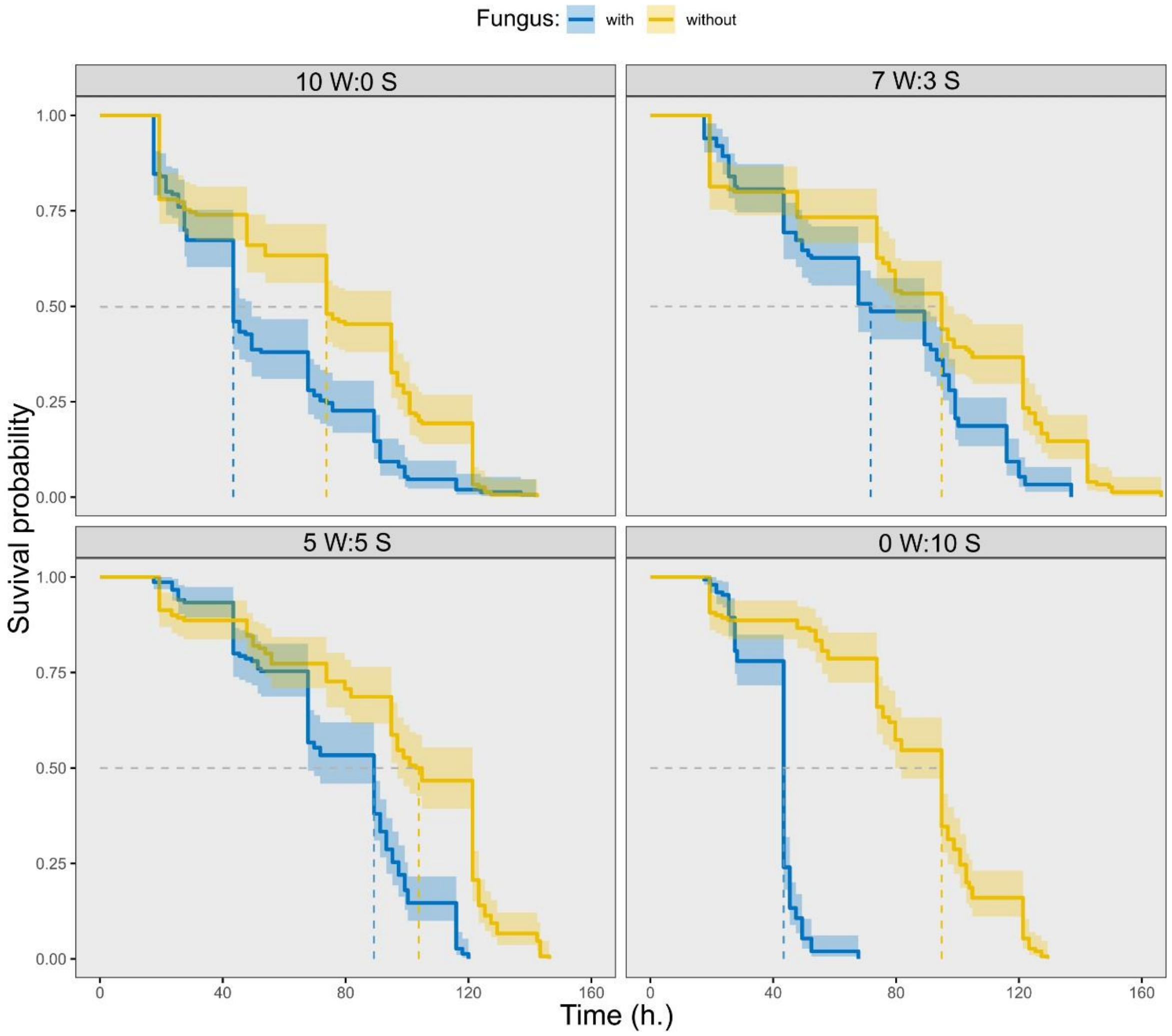

Figure 2. Survival curves of *Nasutitermes corniger* groups composed of different proportions of workers and soldiers (W:S) exposed (blue lines) and non-exposed (yellow lines) to *Metarhizium anisopliae*.

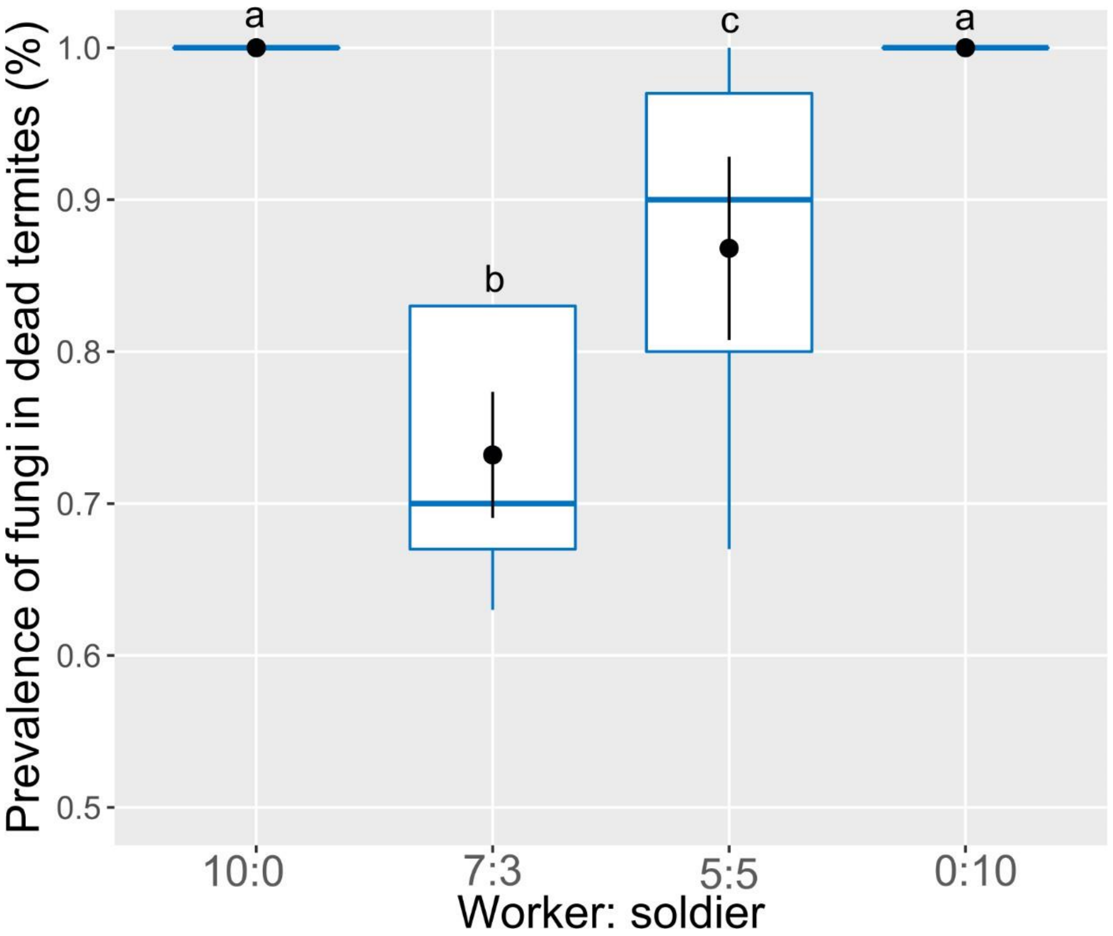

Figure 3. Effect of soldier’s proportion on the prevalence of fungus in dead termite individuals. Boxplots followed by the same letters do not significantly differ by *EMMeans* post-hoc test. The mean values by the black points, blue horizontal lines demarcate the central value of the data set, and vertical lines indicate the minimum and maximum value of the data set.

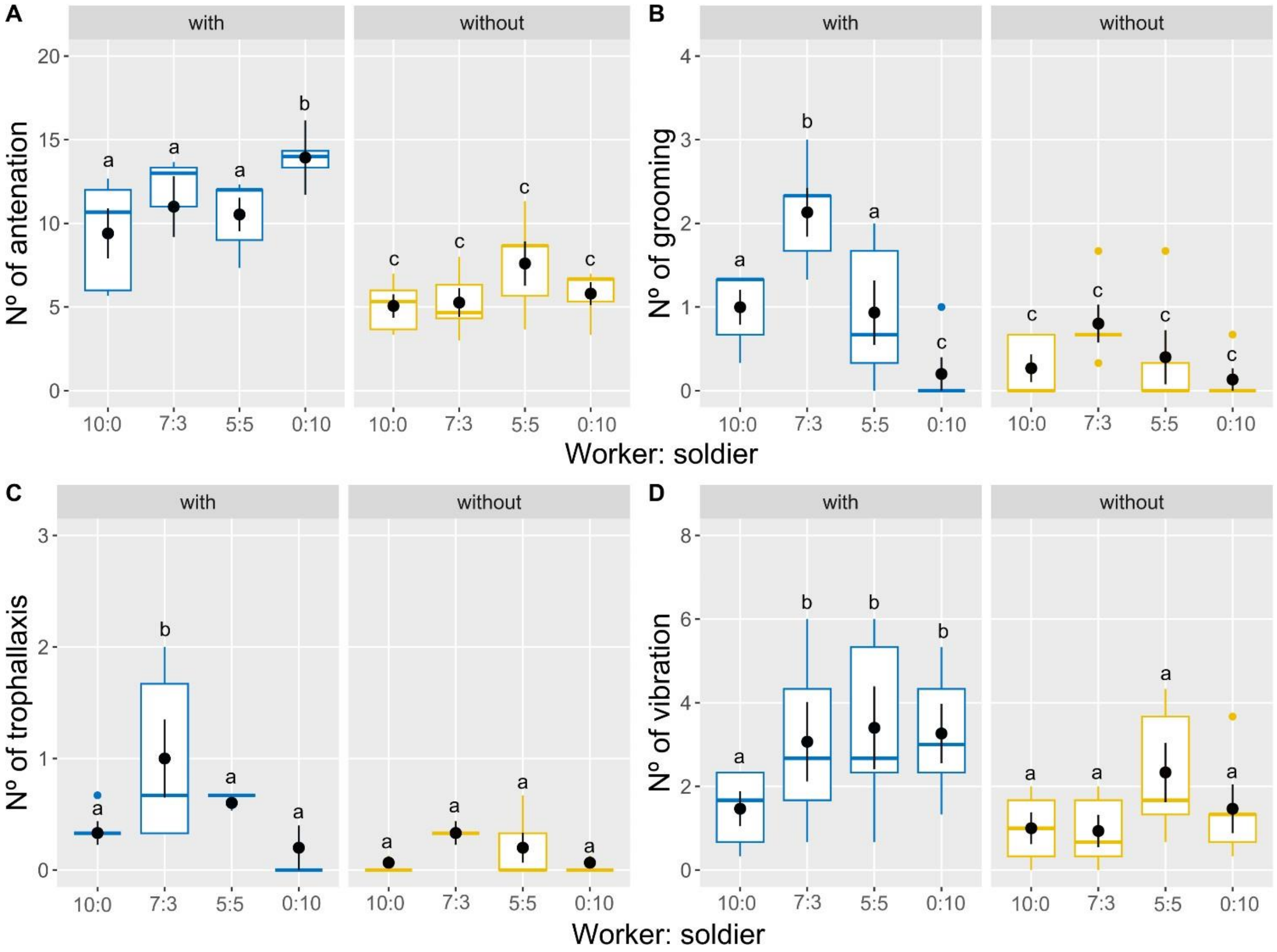

Figure 4. Effects of fungus (with or without) and soldier’s proportion on the behaviour of termite groups. (a) antennation; (b) grooming; (c) trophallaxis; (d) vibration. Boxplots followed by the same letters do not significantly differ by *EMMeans* post-hoc test. Raw data are represented by blue and yellow points, the mean values by the black points, blue and yellow horizontal lines demarcate the central value of the data set, and vertical lines indicate the minimum and maximum value of the data set.

Supporting Information 01. *Nasutitermes corniger* susceptibility to infections by entomopathogenic fungi *Beauveria, Cordyceps* and *Metharizium* genera.

Here, we evaluated the susceptibility of *Nasutitermes corniger* groups composed by workers and soldiers to selected fungal isolates from the species *B. bassiana*, *M. anisopliae* and *C. fumosorosea*. The virulence of each of these strains was tested with an in vitro termite survival assay after exposure to fungal conidia.

Arboreal nests (*N*= 5) of *N. corniger* were collected at the Rural Federal University of Pernambuco (UFRPE) - campus SEDE (8º04'03''S, 34º55'00''W), in the municipality of Recife, state of Pernambuco, Brazil. The climate is characterized as "subtropical humid or sub-humid" (Aw) with an average annual temperature of 25.7 ºC and an average annual precipitation of 988 mm (Climate 2021).

The nests with active colonies of *N. corniger* were removed from the tree and taken to the Laboratory of Insect Ecology at UFRPE to be used for the conduction of bioassays. In the laboratory, colonies inside the nests were placed separately in plastic boxes with moistened cotton. Colonies were kept at laboratory after collection for 24 hours before the bioassays. Termites were individually identified using specific literature. The voucher specimens are deposited in the collection of the Insect Ecology Laboratory - UFRPE.

Conidia preparation. The entomopathogenic fungi *B. bassiana* (strain PL63), *M. anisopliae* (strain ESALQ-E9), and *C. fumosorosea* (strain ESALQ-1296) were obtained from the collection of Entomopathogenic Microorganisms "Prof. Sérgio Batista Alves" of the Laboratory of Pathology and Microbial control (ESALQ/University of São Paulo, Brazil) and streaked onto potato dextrose agar (PDA) plates supplemented with streptomycin (to prevent bacteria contamination). All inoculated plates were incubated for seven days in a climate chamber at 26 °C under a 12L:12D light cycle.

The conidia suspension was prepared by scraping the conidia from plates using a flamed metallic loop. Conidia were then transferred to a 0.05% Tween 80 solution. The conidia suspension was serially diluted and vortexed for five minutes. For conidia counting, 200 μL from of the diluted suspension at 10^{-3} was placed in an improved Neubauer haemocytometer. The conidia spores were counted under a Leica compound microscope at 40x magnification. After the number of conidia in the stock was determined, conidial suspensions for each isolate were prepared in Tween 80 solution at 0.05% and the concentration adjusted to 1.0×10^7 conidia/ml.

Fungal species selection. The virulence test was performed using three entomopathogenic fungi species (*M. anisopliae*, *B. bassiana* and *C. fumosorosea*). To do so, groups with 10 individuals (three soldiers and seven workers) of *N. corniger* were treated with conidial suspensions of each fungus and 0.05% Tween 80 solution (control). Groups of termites were placed in Petri dishes (80 × 15 mm) with a filter paper on the bottom and the 2 ml of the fungal suspensions were sprayed in each plate individually at a distance of 30 cm to reach all termite body. To avoid contamination of controls with conidia, control replicates were consistently set up before the spore-exposure replicates. The Petri dishes were maintained in a climate chamber at 26º C under a 12L:12D light cycle and were observed daily for eight days.

Termite mortality was determined by a lack of a movement reaction. Cadavers were removed daily, and surface was sterilized with 95% alcohol (Gao *et al.* 2012). The cadavers were placed in a wet chamber to confirm mortality by entomopathogenic fungi. Each cadaver was observed to confirm if the fungus outgrowing the insect body matches the applied fungus. The plates were incubated at 26º C under a 12L:12D light cycle until the growth of visible mycelia and the production of conidia on termite carcasses (Rosengaus & Traniello 1997).

We estimated the prevalence of fungi varying from 0 to 1 by dividing the number of termites in the group with fungi by the total number of termites in the group. Three repetitions per treatment (fungal species) and blank control were performed for each of the five colonies tested, totaling 60 repetitions/bioassay and 600 insects.

Data analysis. The statistical analyses were carried out in R statistical software (R Development Core Team 2019) using Generalized Linear Models (GLMs), followed by residual analysis to check suitability of models. Model simplification, when necessary, was conducted by removing non-significant explanatory variables from the initial model and evaluating subsequent changes in the deviance. In all analyses, the average value of each tested colony in each treatment was used as true repetition.

To determine which the most virulent strain (*M. anisopliae*, *B. bassiana* or *C. fumosorosea*), data were submitted to survival analysis using Weibull distribution in order to check whether entomopathogenic fungus (*x-var*) would affect survival of termites (*y-var*).

To check the effect of fungi strains (*x-var*) in mortality rate (*y-var*) per day, data were submitted to Analysis of Deviance (ANODEV, a maximum likelihood equivalent of ANOVA) with Binomial error distribution followed by Contrast Analysis. Tests for each day were conducted separately.

The prevalence of fungi in dead termites was tested through ANODEV with Binomial error distribution followed by Contrast Analysis.

Results

All fungi strains caused mortality and were able to outgrow from *N. corniger* bodies. In general, the mean time to death of termite groups was 2.73 days varying from one to eight days.

The survivorship of *N. coniger* groups was significantly affected by treatments ($\chi^2 = 348.18$, *d.f.* = 3, $P < 0.0001$). Groups of *N. corniger* exposed to *M. anisopliae* survived shorter times than those exposed to *B. bassiana* or *C. fumosorosea* and control (Fig. 1). No significant difference was observed in the survivorship of *N. corniger* groups exposed to *B. bassiana* and *C. fumosorosea* (*P*= 0.29) (Fig. S1).

The percentage of mortality per day of *N. corniger* after infection by different strains of fungi were shown in Table S1. In general, the mortality of 100% of individuals in *N. corniger* groups exposed to *M. anisopliae* occurred in three days while in groups exposed to *B. bassiana* and *C. fumosorosea* occurred only in six and five days, respectively (Table S1).

The prevalence of fungus outgrowth in dead termites was significantly affected by treatments ($F_{3,16}$= 8.27, *P*= 0.001). Termites exposed to fungi strains showed a higher prevalence of fungi compared to control. However, no significant difference was observed among the fungi strains tested (*P*= 0.33) (Table S2).

In general, the results showed that the maximum mortality at three days post-infection in the groups of *N. corniger* treated with *B. bassiana* was 56.0 ± 6% and with *C. fumosorosea* it was 56.0 ± 9%, while for *M. anisopliae* mortality reached 100%. Thus, *M. anisopliae* caused faster mortality with a mean time to death of 1.06 days, as already demonstrated in other higher termite species (Dong *et al.* 2009, Singha *et al.* 2011, Al-Farhani *et al.* 2021). *Metarhizium anisopliae* (ESALQ-E9 strain) was observed as the most virulent strain. Thus, it was selected to be used in the main experiments.

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Table S1. Mortalities (mean per cent ± S.E.) of *Nasutitermes corniger* groups after infection by different strains of fungi.

Strains	**Mortality (%)/time (day)**							
	1 day	2 day	3 day	4 day	5 day	6 day	7 day	8 day
Control	0.26±0.08 a	0.31±0.09 a	0.46±0.13 a	0.66±0.19 a	0.79±0.13 a	0.92±0.05 a	0.99±0.01	1.00±0.00
Beauveira bassiana PL63	0.16±0.04 a	0.35±0.05 a	0.56±0.06 a	0.78±0.08 b	0.98± 0.01 b	1.00±0.00 a	-	-
Cordyceps fumosorosea ESALQ-1296	0.12±0.03 a	0.28±0.06 a	0.56±0.09 a	0.86±0.06 b	1.00±0.0 b	-	-	-
Metarhizium anisopliae ESALQ-E9	0.81±0.09 b	0.96±0.02 b	1.00±0.00 b	-	-	-	-	-

Means followed by the same letters do not significantly differ by ANODEV test followed by Contrast Analysis.

Table S2. Prevalence of fungi strains (mean per cent ± S.E.) of *Nasutitermes corniger* cadavers after bioassays of fungal species selection.

Strains	**Prevalence of fungi in dead termites (%)**
Control	0.00±0.00 a
Beauveira. Bassiana PL-63	0.64±0.013 b
Cordyceps fumosorosea ESALQ-1296	0.58±0.14 b
Metarhizium anisopliae ESALQ-E9	0.47±0.13 b

Means followed by the same letters do not significantly differ by ANODEV test followed by Contrast Analysis

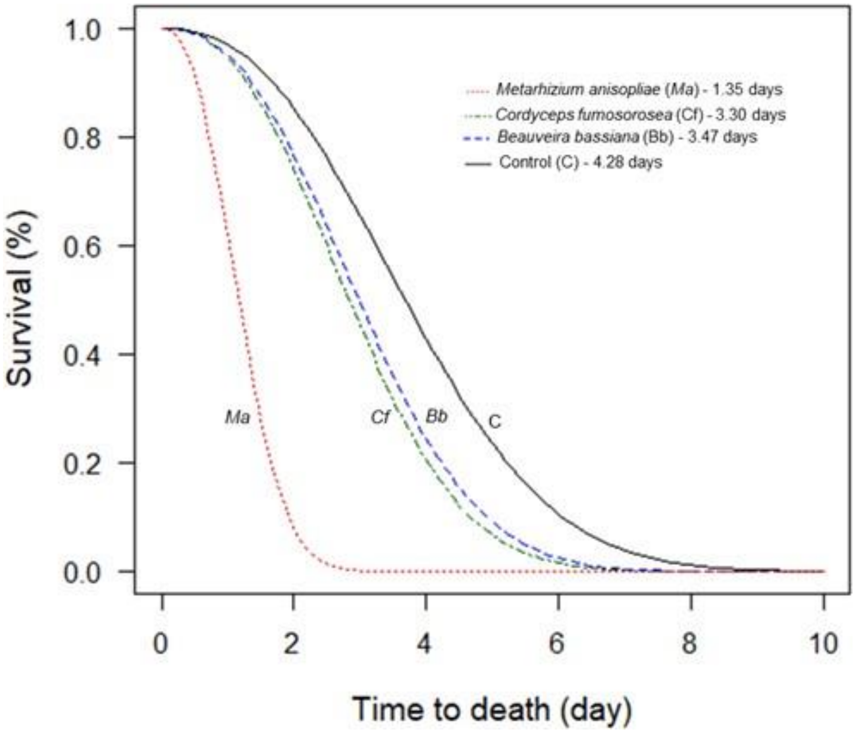

Figure S1. Survival curves for *Nasutitermes corniger* groups exposed to three different fungi strains and control.

Supporting Information 02. Correlation plot and Pearson's correlation matrix for behavior observed in group with and without fungus

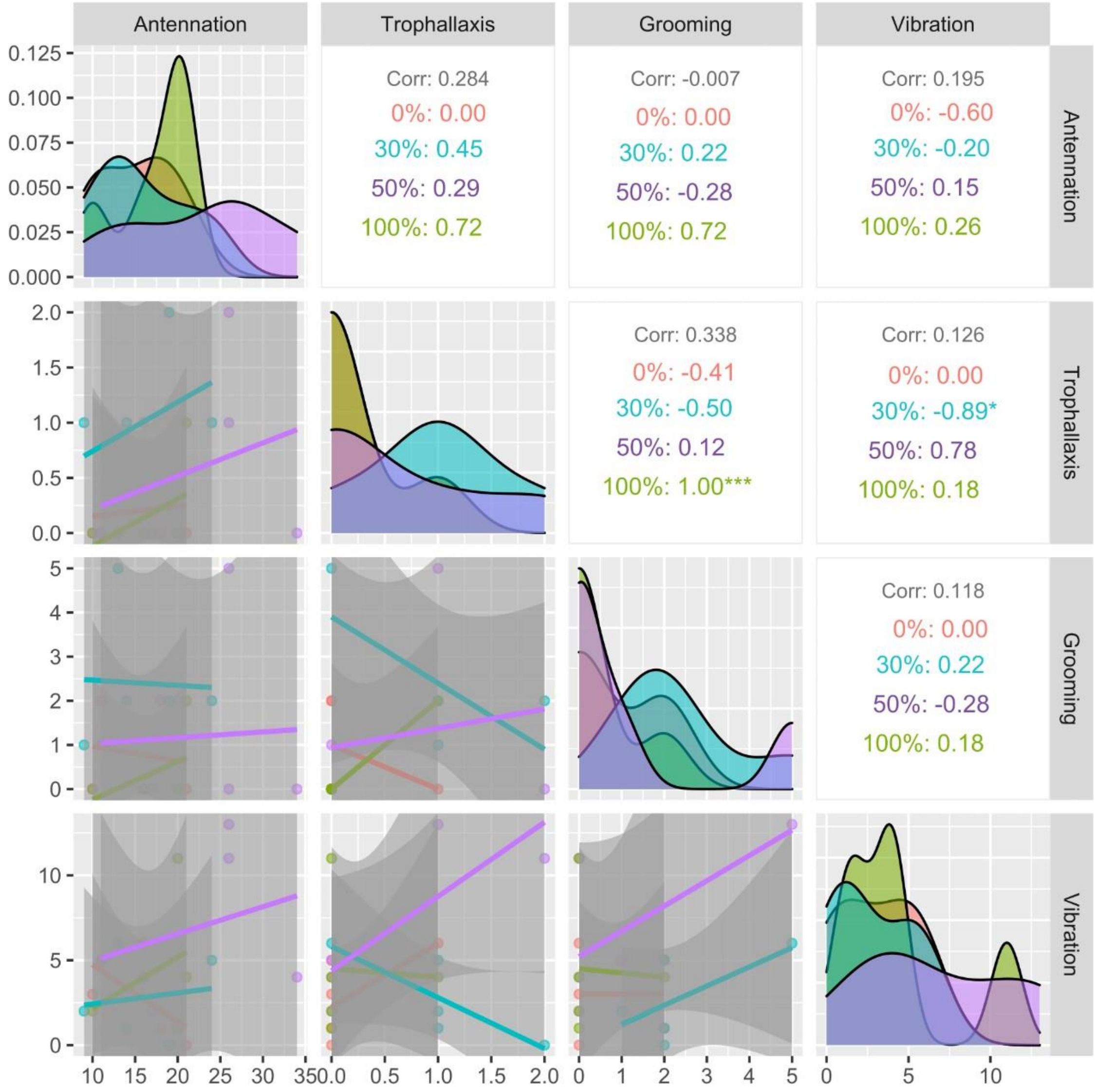

Figure S2. Distribution plot, correlation plot, and Pearson's Correlation Coefficient Matrix for comparisons among behaviors by caste composition in the non-exposed termite group (control); the upper panel above the diagonal shows Pearson's Correlation Coefficients. The lower panel below the diagonal gives their correlation plots. The diagonal show distribution plots.

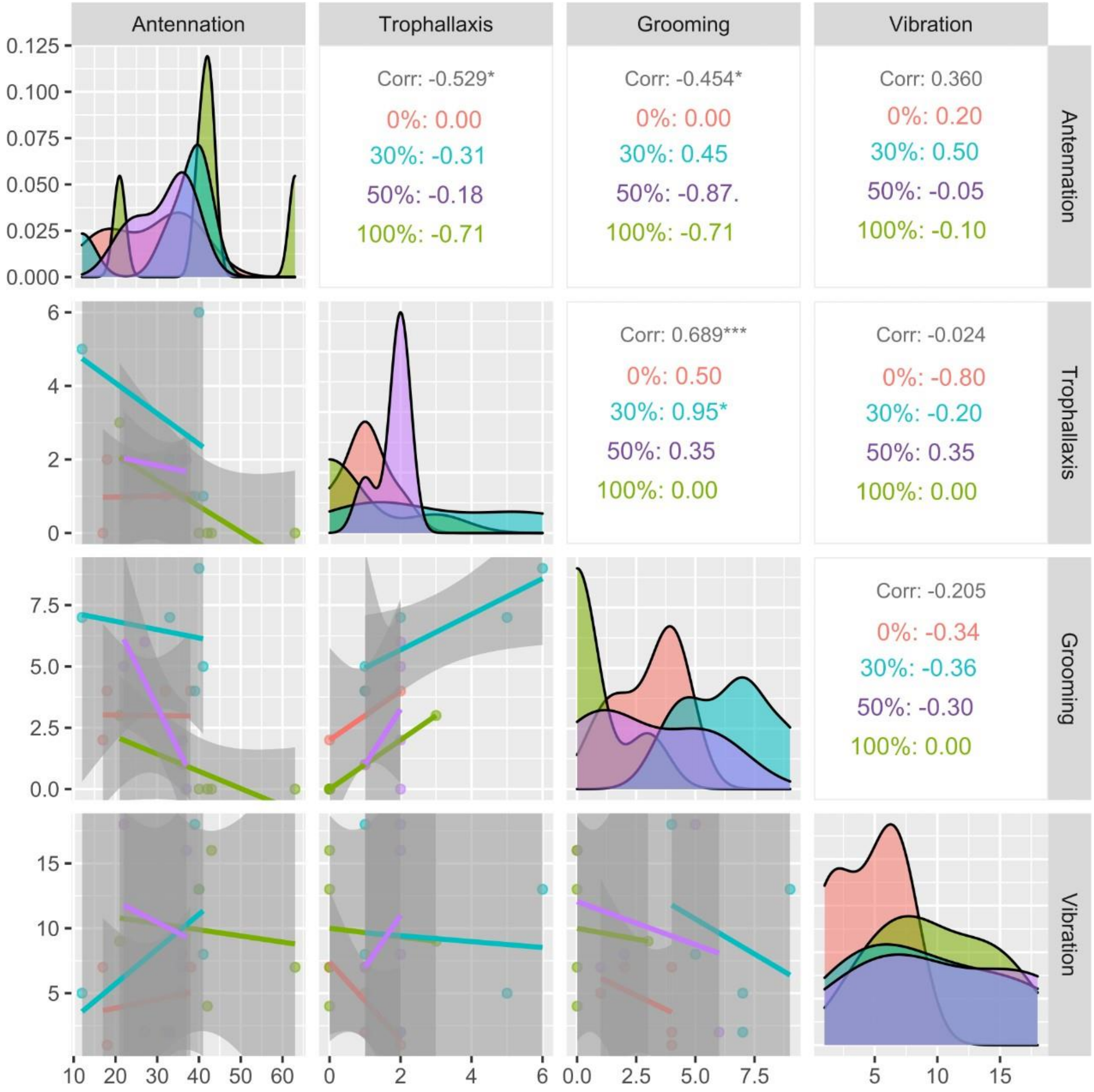

Figure S3. Distribution plot, correlation plot, and Pearson's Correlation Coefficient Matrix for comparisons among behaviors by caste composition in the exposed termite group (control); the upper panel above the diagonal shows Pearson's Correlation Coefficients. The lower panel below the diagonal gives their correlation plots. The diagonal show distribution plots.

CHAPTER 3

SECRETIONS OF THE FRONTAL GLAND OF A NASUTE TERMITE SOLDIER: THE ROLE IN THE DEFENSE AGAINST AN ENTOMOPATHOGENIC FUNGI

LARISSA FREITAS FERREIRA[1,2], THIAGO H.C. MENDONÇA[1,2], ALINE N.F. SILVA[1,2], SARA Y.M. WATANABE[1,2], CAMILA C. MOREIRA[3], PAULO FELLIPE. CRISTALDO[1,2]

[1] Post Graduate Program in Entomology, Department of Agronomy, Universidade Federal Rural de Pernambuco, Recife, Brazil

[2] Laboratory of Insect Ecology, Department of Agronomy, Universidade Federal Rural de Pernambuco, Recife, Brazil

[3] Koppert Biological Systems, Piracicaba, Brazil

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ABSTRACT – The eusocial insects are characterized by an efficient division of labor. Among these insects, termites have evolved specialized defensive individuals (soldiers). Because to the high energy cost to their production and they are incapable of feeding themselves, soldiers were seen as a “burden”, but studies have been shown that the play a crucial role in other activities, such as colony immunity. Soldiers of the subfamily Nasutitermitinae have frontal glands that secret defensive substances. The contribution of soldiers and these substances to colony immunity is little known. To investigate the effect of soldiers of *N. corniger* on the survival of infected workers survival bioassays were performed in Petri dishes with *N. corniger* groups were placed in different combinations (10 infested workers; 10 healthy workers; 7 infested workers and 3 healthy soldiers). To investigate the effect of the frontal gland secretion on the survival workers infected we carry out the following treatments: (1) 10 workers with 0.05% Tween 80 solution (control); (2) 10 workers with fungal suspension; and (3) 10 workers with fungal suspension and frontal gland extracts. To evaluate the effect of direct contact of the fungus with frontal gland secretion, frontal gland extract was placed in a Petri dish prepared with conidia suspension. Overall, workers of *N. corniger* infected with *Metarhizium anisopliae* survive less than those with soldiers or uninfected (control). The groups of *N. corniger* workers exposed to *M. anisopliae* survived longer when there was the presence of the soldier's frontal gland compared to groups with only fungi, and the secretions of soldiers have an inhibitory effect on the growth of *M. anisopliae*. This work contributes to a better understanding of the function of the soldiers, and defense mechanisms that involve chemical secretions.

KEYWORDS: antifungal substance, defensive secretion, frontal gland, *Nasutitermes corniger*

SECREÇÃO DA GLÂNDULA FRONTAL DE UM SOLDADO DE CUPIM NASUTO: O PAPEL NA DEFESA CONTRA FUNGO ENTOMOPATOGÊNICO

RESUMO – Os insetos eussociais são caracterizados por uma divisão eficiente do trabalho. Entre esses insetos, os cupins desenvolveram indivíduos defensivos especializados (soldados). Devido ao alto custo energético para sua produção e à incapacidade de se alimentarem, os soldados eram vistos como um “fardo”, mas estudos têm demonstrado que desempenham um papel crucial em outras atividades, como a imunidade das colônias. Os soldados da subfamília Nasutitermitinae possuem glândulas frontais que secretam substâncias defensivas. A contribuição dos soldados e dessas substâncias para a imunidade das colônias é pouco conhecida. Para investigar o efeito dos soldados de *N. corniger* na sobrevivência de operários infectados, bioensaios de sobrevivência foram realizados em placas de Petri com grupos de *N. corniger* colocados em diferentes combinações (10 operários infestados; 10 operários saudáveis; 7 operários infestados e 3 soldados saudáveis) Para investigar o efeito da secreção da glândula frontal na sobrevivência dos operários infectados realizamos os seguintes tratamentos: (1) 10 operários com solução de Tween 80 a 0,05% (controle); (2) 10 operários com suspensão fúngica; e (3) 10 operários com suspensão fúngica e extratos de glândula frontal. Para avaliar o efeito do contato direto do fungo com a secreção da glândula frontal, o extrato da glândula frontal foi colocado em placa de Petri preparada com suspensão de conídios. No geral, os operários de *N. corniger* infectados com *Metarhizium anisopliae* sobrevivem menos do que aqueles com soldados ou não infectados (controle). Os grupos de operários de *N. cornige*r expostas a *M. anisopliae* sobreviveram mais quando houve a presença da glândula frontal do soldado em comparação aos grupos apenas com fungos, e as secreções dos soldados têm efeito inibitório sobre o crescimento de *M. anisopliae*. Este trabalho contribui para uma melhor compreensão da função dos soldados e dos mecanismos de defesa que envolvem secreções químicas.

PALAVRAS-CHAVE: comportamento, *Metarhizium anisopliae*, *Nasutitermes corniger*, sobrevivência

Introduction

Life on Earth evolved through rare but enormous stages known as ‘major evolutionary transitions’ (West *et al.* 2015). According to Bernadou *et al.* (2021), the evolution of eusociality in insects (*e.g.* termites, ants and some species of bees and wasps) has been considered an important evolutionary transition. Eusociality is characterized by the overlapping of generations, reproductive division of labor, and brood care (Wilson 1971). The division of labor between reproductive individuals (queens, and in termites also kings) and non-reproductive individuals (workers and soldiers) is unquestionably a major characteristic of eusocial insects (Korb 2016).

In general, two patterns of division of labor among non-reproductive individuals are known: caste polyethism and age polyethism (Wilson 1971). Caste polyethism is defined as performing tasks based on individual’s caste, which individuals present changes in shape and body proportions and/or behavioral specialization according to tasks that they perform. The defensive caste, for example, present morphological apparatus such as prominent mandibles and/or an impressive chemical weapon. On the other hand, age polyethism is the division of tasks based on age, is common among workers, with older one performing riskier tasks compared to young workers. That is, young individuals are performing tasks within the nest, such as caring for the brood and the reproductive, while older ones perform riskier tasks, such as foraging outside the nest (Crosland & Traniello 1997). In addition, termites can also exhibit sex polyethism, when male and female workers perform different tasks, for example, in *Hodotermopsis japonica* males change tasks from gnawing food (as minor workers) to defense (as soldiers), whilst females specialize in foraging (Miura *et al.* 1998).

Termites evolved eusociality independent from Hymenoptera. Nowadays, these insects are known as “social cockroaches”, a monophyletic clade nested within the Blattodea order, with *Cryptocercus* woodroaches as sister taxon (Engel *et al.* 2009). Eusocial lineages differ significantly in the evolution of a sterile caste. In termites, the soldier caste was the first sterile caste to evolve and remains the only truly sterile caste in many species (Tian & Zhou 2014). For decades, soldiers were seen as a “burden” for termite colonies, since these individuals were highly costly in terms of energy to their production and they are trophically dependent on workers (Haverty 1977, Sacramento *et al*. 2020). However, in the last years, a plenty of studies have been shown that the role of termite soldiers goes beyond defending the colonies, mainly in Nasutitermitinae subfamily. In *Nasutitermes corniger* (Motschulsky) (Termitidae: Nasutitermitinae), for example, soldiers increase the ability colonies in exploiting food resources (Sacramento *et al.* 2020). In *N. costalis* and *N.* aff. *coxipoensis*, soldiers significantly contribute in the initial phase of food resource selection (Traniello 1981, Almeida *et al.* 2016).

Interestingly, soldiers also seem to play a crucial role in the social immunity of termite colonies. Recently, has been shown that groups of *N. corniger* with a natural proportion of soldiers (*i.e.,* proportion of soldiers observed in colonies in nature - 30%) infected with *Metarhizium anisopliae* (Metsch.) exhibit a higher number of prophylactic behaviors (*e.g.* grooming and trophallaxis), reducing the number of infected termites and consequently the effect of the entomopathogen on termite survival (Ferreira *et al.* 2023). In addition, alarm communication through vibrational movements were more consistent when the soldier was present, indicating that this caste perceives the pathogen and transmits the information to other individuals in the group.

In termites, the alarm communication about presence of threats is transmitted by alarm pheromones that are present in the soldiers' frontal gland of species from Rhinotermitidae, Serritermitidae, and Termitidae families (Noirot & Darlington 2000, Costa-Leonardo *et al.* 2023). However, it is not only pheromones that are secreted by the frontal glands (Prestwich 1983), it likely secretes other substances that may have an inhibitory effect on bacterial and fungal growth. (Rosengaus *et al*. 2000, Fuller 2007). Previous studies revealed that (−)-β-elemene secreted by soldiers of *R. speratus* suppress the growth of entomopathogenic fungi, *M. anisopliae* and *Beauveria bassiana* (Mitaka *et al.* 2017). Additionally, α-pinene and limonene from *Nasutitermes* species, exhibit antifungal and antibacterial activities; as well as trinervitane and n-hexanoic acid from *Zootermopsis* species (Rosengaus *et al.* 2000, 2004, Zhao *et al*. 2004). Therefore, the secretion produced in the soldier's frontal gland seems to have a number of functions in addition to the defensive ones, such as fungicidal activity (Fuller 2007).

Species belonging to Nasutitermitinae subfamily developed rather long "nasus", which has the function of expelling the defensive secretion (Santos & Costa-Leonard 2006). This defensive secretion is usually a mixture of mono- and diterpenes (Prestwich 1979, 1983). The soldiers of the genus *Nasutitermes* (*i.e.,* nasute soldiers) have rather reduced mandibles and a well-developed "nasus" (front tube) (Miura & Matsumoto 2000, Costa-Leonardo & De Salvo 1987). The principal component of the secretion of the frontal glands of *Nasutitermes* is α-pinene (30-97% of the secretion). Secondary components are β-pinene (1-64%) and limonene (0-30%) (Moore 1964, Gush *et al.* 1985, Prestwich 1979, Cruz *et al.* 2014, Mello *et al.* 2016, Oliveira-Neto *et al.* 2022). It is known assumed that the terpenes secreted by soldiers are used as antimicrobial substances (Gershenzon & Dudareva 2007). On the other hand, to the best of our knowledge, no studies have observed this in *N. corniger,* a common species in the Neotropical region of South America (Constantino 2002).

Thus, we aimed to investigate the effect of the frontal gland secretion of *N. corniger* on the survival workers infected by *M. anisopliae* and, in addition, we will examine the effect of the secretion in fungal growth. Specifically, we tested the following hypothesizes: (1) the presence of healthy soldiers positively affects the survival of infected workers; (2) the frontal gland extract increases the survivorship of *N. corniger* workers infected with the fungus; and (3) the growth of *M. anisopliae* fungus is negative affects by contact with frontal gland extract of *N. corniger* soldiers.

We selected the species *M. anisopliae* because studies of virulence and defense mechanisms in termites were performed with *M. anisopliae* as a model entomopathogen (Chouvenc *et al.* 2009, Rosengaus *et al.* 2011, Singha *et al.* 2011, Ambele 2020, Ferreira *et. al.* 2023).

Material and Methods

Termite collection and ethics statement Colonies of *N. corniger* were collected at the campus “SEDE” of the Rural Federal University of Pernambuco (UFRPE) (8º04’03’’S, 34º55’00’’W), in the municipality of Recife, state of Pernambuco, Northeastern Brazil. According to Köppen classification, the climate in the area is characterized as “subtropical humid or sub-humid” (Aw type), with an average annual temperature of 25.7 ºC and an average annual precipitation of 988 mm (Climate 2024).

Arboreal nests with active colonies of *N. corniger* were removed from the trees and taken to the Laboratory of Insect Ecology at UFRPE to be used to prepare chemical extracts and in bioassays. In the laboratory, colonies were placed separately in plastic boxes with moistened cotton. Colonies were kept in the laboratory after being taken from the field for 24 hours before the bioassays. A total of eight colonies were sampled to conduct bioassays. All bioassays were performed under controlled conditions (25 ± 2 ºC, 70 ± 2% U.R., and low light conditions).

Termite collections were carried out with permission of ICMBio – IBAMA to PFC (#47652). No specific permits were required for the described studies undertaken in the laboratory with a non-endangered or protected species.

Conidia preparation. The entomopathogenic fungus *M. anisopliae* (strain ESALQ-E9) was obtained from the collection of Entomopathogenic Microorganisms "Prof. Sérgio Batista Alves" of the Laboratory of Pathology and Microbial control (ESALQ/University of São Paulo, Brazil) and streaked onto potato dextrose agar (PDA). All inoculated plates were incubated for seven days in a climate chamber (Marconi - MA403) at 26 °C under a 12L:12D light cycle.

The conidia suspension was prepared by scraping the conidia from the plates using a flamed metal loop. The conidia were transferred to a 0.05% Tween 80 solution, and subsequently, the suspension was serially diluted and shaken (vortexed) for five min. For conidia counting, 200 μL of the suspension diluted to 10^{-3} was placed in a Neubauer haemocytometer. The spores were counted under a Leica compound microscope. Finally, the final suspension was prepared in a Tween 80 solution at 0.05% and the concentration adjusted to 1.0×10^{7} conidia/mL (Ferreira *et al.* 2023).

Preparation of frontal gland extracts. Extracts from frontal gland of soldiers were prepared following protocol described in Cristaldo *et al.* (2015). Briefly, extracts were prepared from 50 soldier heads (nasus cut off) submerged into hexane solvent (5 μl per head), macerated overnight at 4°C followed by another washing with 5-10 μl of hexane per head. Final samples were stored at −18°C and used for bioassays, in which we always worked with recounted soldiers equivalent (1 individual extracted in known solvent volume). For each colony sampled (N= 8), three frontal gland extracts were prepared, totalizing 24 extracts.

In termites, frontal gland is an exocrine gland present in soldiers, imagoes, and workers, being more developed in soldiers (Šobotník *et al.* 2010, Costa-Leonardo *et al.* 2023). Thus, we used worker head extracts as control in the antifungal assays. Such procedure was carried out in order to ensure that possible inhibitory effects in the development of the fungus were not from another substance present in the head of termites. The workers head extracts (WHE) were prepared following the same procedures and conditions described in the preparation of frontal gland extracts (see above).

Experimental procedures

Does the presence of healthy soldier reduce the effect of fungus on workers survivorship? In order to check whether the presence of heathy soldiers contributes to decrease the negative effect of fungi on the survivorship of *N. corniger* workers, survival bioassays were conducted with groups of 10 individuals in the following treatments: (1) 10 heathy workers (control), (2) 10 infested workers, and (3) seven infested workers and three healthy soldiers (Fig. 1A). The choice of the caste ratio and termite numbers in each experimental arena was a compromise between natural caste proportions (soldier:worker=3:7; Thorne & Breisch 1985) and optimal density to maximize inter-individual contacts and survival (Miramontes & Desouza 1996). Only healthy and non-injured individuals were used. For each of the treatments, three repetitions were carried out for each of the eight colonies tested, totalizing 72 experimental units.

Workers of *N. corniger* were treated with 1.0 x 10^7 conidia/ml of *M. anisopliae* suspended in 0.05% Tween 80 or a 0.05% Tween 80 solution (control). The fungal suspension (2 ml) was sprayed on each plate individually at a distance of 30 cm to reach all termite bodies (Ferreira *et al.* 2023). Control replicates were consistently set up before the spore-exposure replicates to avoid contamination of controls with conidia. Following exposure, workers were transferred to a new sterile Petri dish (80 x 15 mm), whose internal bottom surface was lined with filter paper (Whatman nº 1; Ø 60 mm). The Petri dishes were kept under controlled conditions (26ºC, 70% of relative humidity, and darkness) in a climate chamber (Marconi - MA403).

The mortality evaluations were carried out at 15-min intervals until completing 2 h of observation, on the first day. Then, we evaluated out at 1-h intervals, for a total of ten hours of evaluation. The day after, we evaluated out at 2-h intervals until the death of all individuals. The termite mortality was determined by a lack of a movement reaction.

Cadavers were removed daily, and the surface was sterilized with 95% alcohol (Gao *et al.* 2012). The cadavers were placed in a wet chamber to confirm mortality by entomopathogenic fungi. The presence of the fungus was confirmed by the visual observation of the withe hyphae and green masses of conidia, following procures described in Ferreira *et al.* (2023).

Does the frontal gland extract mitigate the effect of fungus on workers survivorship? To determine whether frontal gland extract mitigate the effect of fungus on survivorship of *N. corniger*, survival bioassays were conducted with groups of 10 workers in the following treatments: (1) uninfected workers (control); (2) uninfected workers + frontal gland extract (extract control); (3) infected workers; and (4) infected workers + frontal gland extract (Fig. 1B). The choice of 10 individuals of termites in the arenas was a compromise with optimal density to maximize inter-individual contact and survival (Miramontes & DeSouza 1996). Only healthy and non-injured individuals were used. A total of three repetitions were performed for each combination of treatments (N = 4) x colony (N = 8), totalizing 96 bioassays.

Survival bioassays were conducted in experimental arenas made from Petri dish (80 × 15 mm); whose internal bottom surface was lined with filter paper (Whatman nº 1; Ø 60 mm). In this bioassay, *N. corniger* workers were exposed to spores in solution by allowing them to walk freely in the filter paper disk that had been impregnated with 8 μl of spore solution (1.0 x 10^7 conidia/ml) using a micropipette (Hamilton®). The treatments containing frontal gland extracts were also applied onto the filter paper using a micropipette (Hamilton®). The gland equivalents (gl./Eq.) for the treatment were established in 8 μl of extract (equivalent to two frontal glands). Control of bioassays consisted of the 8 µl 0.05% Tween 80 solution. Control replicates were consistently set up before the spore-exposure replicates to avoid contamination of controls with conidia. The Petri dishes were kept and maintained in a climate chamber (Marconi - MA403) at 26°C under and darkness.

The mortality evaluations were carried out at 15-min intervals until completing 2-h of observation and then 1-h intervals until the death of all individuals. The termite mortality was determined by a lack of a movement reaction. Cadavers were removed daily, and the surface was sterilized with 95% alcohol (Gao *et al.* 2012). The cadavers were placed in a wet chamber to confirm mortality by entomopathogenic fungi following procures described in Ferreira *et al.* (2023).

Does the frontal gland extract inhibit the development of fungus? In order to check whether frontal gland secretion of *N. corniger* soldiers inhibit fungal growth, antifungal assays were conducted in the following conditions: (1) only fungus; (2) fungus + Tween 80 ('Tween control'); (3) fungus + worker head extract (WHE) ('extract control'); and (4) fungus + frontal gland extract (FGE) (Fig. 1C). The 'FGE' treatment provides mostly terpenoids compounds (Sobotnik *et al.* 2010, Mello *et al.* 2016). The 'WHE' extract control provides mainly hydrocarbons and no terpenoids since workers do not have a frontal gland; being an extra 'control' (see Cristaldo *et al.* 2015 for details).

Antifungal assays were conducted in a PDA plates (80 × 15 mm), which were seeded with 8 µl of a 1.0×10^7 conidia/ml suspension of the fungus *M. anisopliae*. Tween 80 (8 µl of 0.05% Tween 80 solution), WHE (8 µl) and FGE (8 µl) were applied using a micropipette (Hamilton®). All plates were wrapped with two layers of Parafilm (Life Clean®) and maintained in a climate chamber (Marconi - MA403) at 26 ºC under and darkness.

After seven days, all plates were photographed using a digital camera (D7500 Nikon, Tokyo, Japan). To measure the size of mycelia (area in cm2), images were uploaded at ImageJ® software (Schneider *et al.* 2012); and the total visible area was measured, selecting all *M. anisopliae* growth.

For each treatment, three repetitions were performed for each colony (N = 8), totalizing 96 bioassays.

Statistical analyses. Statistical analyses were carried out in R software (R Development Core Team 2024), using Generalized Linear Mixed Models (GLMMs), followed by residual analysis to check model assumption and model quality. To assess the significance of treatment effects, we performed a Wald test. Pairwise comparisons of estimated marginal means (EMMeans) corrected by the false discovery rate method (Lenth 2023) were used as post-hoc tests.

Data from survival bioassays were initially submitted to survival analysis using a Weibull error distribution to obtain the mean time to death (hours), as described in DeSouza *et al.* (2009) and Ferreira *et al.* (2023). In this analysis, the mean time to death of all individuals in the experimental arena was calculated for the nests submitted to each one of treatments. Then, to check the effect of treatments (x-var) on the mean time to death of *N. corniger* (y-var), data were submitted to GLMM under Gaussian error distribution. Repetitions and the colony identity were used in the models as random factors.

To verify the effect of frontal gland extract (x-var) on fungal growth (y-var), data were submitted to GLMM with Gaussian error distribution. Repetitions and the colony identity were used in the models as random factors. All graphs were generated with ggpubr package (Kassambara 2023).

Results

Does the presence of healthy soldier reduce the effect of fungus on workers survivorship? The mean time of death *N. corniger* workers was significantly affected by treatments (GLMM; Chisq = 39.06, *d.f.* = 2, P = 0.02). In general, workers of *N. corniger* infected with *M. anisopliae* survive less time than those with soldiers or uninfected (control) (Fig. 2A). There is no significant difference between groups with infected workers + healthy soldiers and groups with only heathy workers (control) (Tab. 1A). Survival curves showing the dynamic of death in groups are shown in Figure 3A.

Does the frontal gland extract mitigate the effect of fungus on workers survivorship? There is a significant effect of treatments on mean time to death of *N. corniger* workers (GLMM; Chisq = 49.83, *d.f.* = 3, P < 0.01). Groups with *N. corniger* workers submitted to ‘fungus + FGE’ and control (Tween 80%) survived longer than *N. corniger* workers submitted to ‘FGE’ followed by those with only fungus (Fig. 2B, Tab. 1B). Biologically, fungus-contaminated *N. corniger* workers are able to inhibit the effect of the fungus through the secretion of the soldiers' frontal gland and survive for the same length of time as uninfected individuals. Our results also showed that it is not the secretion from the soldiers' frontal gland alone that increases survival, but the combination of a pathogen and the secretion. Survival curves showing the dynamic of death in groups are shown in Figure 3B.

Does the frontal gland extract inhibit the development of fungus? The growth area of *M. anisopliae* fungus was significantly affected by treatments (GLMM; Chisq = 72.47, *d.f.* = 3, P < 0.001). In general, FGE decrease the area of fungus compared to other treatments (Fig. 4). There is no significant effect between fungus and ‘fungus + WBE’ (Tab. 1C). The most representative images of the mycelia of *M. anisopliae* are shown in Figure 5.

Discussion

Eusocial insects employ multi-defense strategies against fungal infections. Similar to biological cells, individuals of eusocial groups carry out protective tasks such as pathogen recognition, chemical communication, pathogen killing by burial ("social encapsulation"), and/or antifungal secretions (similar to humoral immunity) and active social exclusion (Schmid-Hempel 1998, Cremer *et al.* 2007, Rosengaus *et al.* 2011, Liu *et al.* 2019). Interestingly, antifungal secretions frequently act in concert with behavioral responses (*e.g.* trophallaxis), which helps to the social spread of antifungal defense secretions in fungus-infected individuals. In the present study, we analyzed the importance of nasute soldier and the secretion of its frontal gland in the survival of workers infected by the fungus. In general, our results showed that the presence of healthy soldiers increased the mean time to death of infected workers; such positive effect on survivorship of workers seems to be related with defensive secretions of *N. corniger* soldiers against fungal infection.

The defensive secretion produced in the frontal gland of soldiers from Rhinotermidae, Serritermitidae, and Termitidae typically includes a mixture of mono- and diterpenes as well as sesquiterpenes (Prestwich 1979, 1983, Prestwich & Collins 1981; Šobotník *et al.* 2010). Among the various roles of the frontal gland secretion, the alarm pheromone, the production of viscous solutions to impede the movements of predator, caste regulation, and immunity (antifungal and antibacterial properties) are noteworthy (Prestwich & Collins 1981, Everaerts *et al.* 1988, Rosengaus *et al.* 2000, Fuller 2007, Tarver *et al.* 2009, Šobotník *et al.* 2010). The role of the frontal gland secretion of *N. corniger* soldiers in the immunity against fungal infection observed in the present study is probably caused by the monoterpenes α-pinene, β-pinene and limonene, once

Rosengaus *et al.* (2000) showed that major components of frontal glad from *N. costalis* (*i.e.*, synonymy *N. corniger*) and *N. nigriceps* inhibits the germination of *M. anisopliae*.

In contrast to our results, Rosengaus *et al.* (2000) observed that *N. costalis* and *N. nigriceps* workers exposed to *M. anisopliae* showed no survival advantage when combined with soldiers. Interestingly, infected workers groups that were combined with healthy soldiers did not have a different survival when compared to healthy workers (control), differing only from the group of infected workers (Fig. 2). This is evidence that soldiers can be effective in mitigating the effects of an infection, thus improving infected workers survival rates. Therefore, soldiers contribute to the survival of the workers living with them through the production of antifungal chemicals or volatiles.

Understanding the significance of chemical compounds secreted by soldiers is an important part of the defensive system against pathogens, which involves ecological aspects (*i.e.*, microhabitat and nutritional niche) in connection with internal and external immune defense. External immune defenses include behavioral mechanisms, for example, grooming that contribute in the spread of antimicrobial secretions among nestmates, reducing pathogen infections (Otti *et al.* 2014, Ferreira *et al.* 2023). Therefore, antimicrobial secretions may have been a major role in the evolution of termite's immune systems. The presence of antimicrobial activity in several lineages of termites suggests that an immunological role for soldiers is a conserved attribute of the ancestral trait of termites, as observed with GNBP2 in *M. darwiniensis* oral secretions and tGNBP-2 in *N. corniger* (Bulmer *et al*. 2009, He *et al.* 2018). According to Bulmer *et al*. 2012, an ancestral GNBP appears to have been duplicated in a common ancestor of subsocial *Cryptocercus* woodroaches and termites. In addition, GNBP2 has been shown to be a key regulator of social immunity in termites and, it is evolutionarily conserved throughout the Isoptera (Termitoidea clade) (Bulmer *et al.* 2009, Bulmer & Crozier 2004).

In conclusion, the results obtained in the present study highlight the importance of the frontal gland secretion from nasute soldiers in inhibiting fungal infections. The compounds produced in the frontal gland of *N. corniger* enhance the survivorship of workers after exposure to fungus, which significantly contributes to colony-level immunity. This study may contribute to a better understanding of soldier function in termite colony as well as the use of chemical defenses in inhibiting fungal infections. In addition, we intend to stimulate further research about the evolution of immune defenses, and the role of soldiers in the termite colonies.

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Table 1. Summary of post-hoc test (EMMeans) testing the effect of of termite's soldiers and its frontal gland extracts on survival of *Nasutitermes corniger* workers and on growth of *Metarhizium anisopliae* fungus. FGE: frontal gland extract. WHE: workers head extracts.

Treatment	**EMMeans**	**SE**	***d.f.***	**Confidential interval**		**Group***
				Lower	**Upper**	
A) tmm ~ treatment + (1 | repetition/colony)						
Healthy workers	23.9	1.42	9,63	19.8	28.0	a
Infected workers	22.0	1.42	9,63	17.9	26.1	b
Infected workers + Healthy soldiers	22.1	1.42	9,63	18.0	26.2	b
B) tmm ~ treatment + (1 | repetition/colony)						
Control	23.0	1.05	31,2	20.3	25.8	a
FGE	21.7	1.05	31,2	19.0	21.4	b
Fungus	20.6	1.05	31,2	17.8	22.5	c
Fungus + FGE	23.0	1.05	31,2	20.2	25.7	c
C) area ~ treatment + (1 | repetition/colony)						
Fungus	13.40	1.4	9,77	9.13	17.7	a
WHE	12.74	1.4	9,77	8.47	17.0	a
Tween 80	10.52	1.4	9,77	6.25	14.8	b
FGE	6.48	1.4	9,77	2.21	10.7	c

* Group with the same letter did not differ statistically ($p > 0.05$).

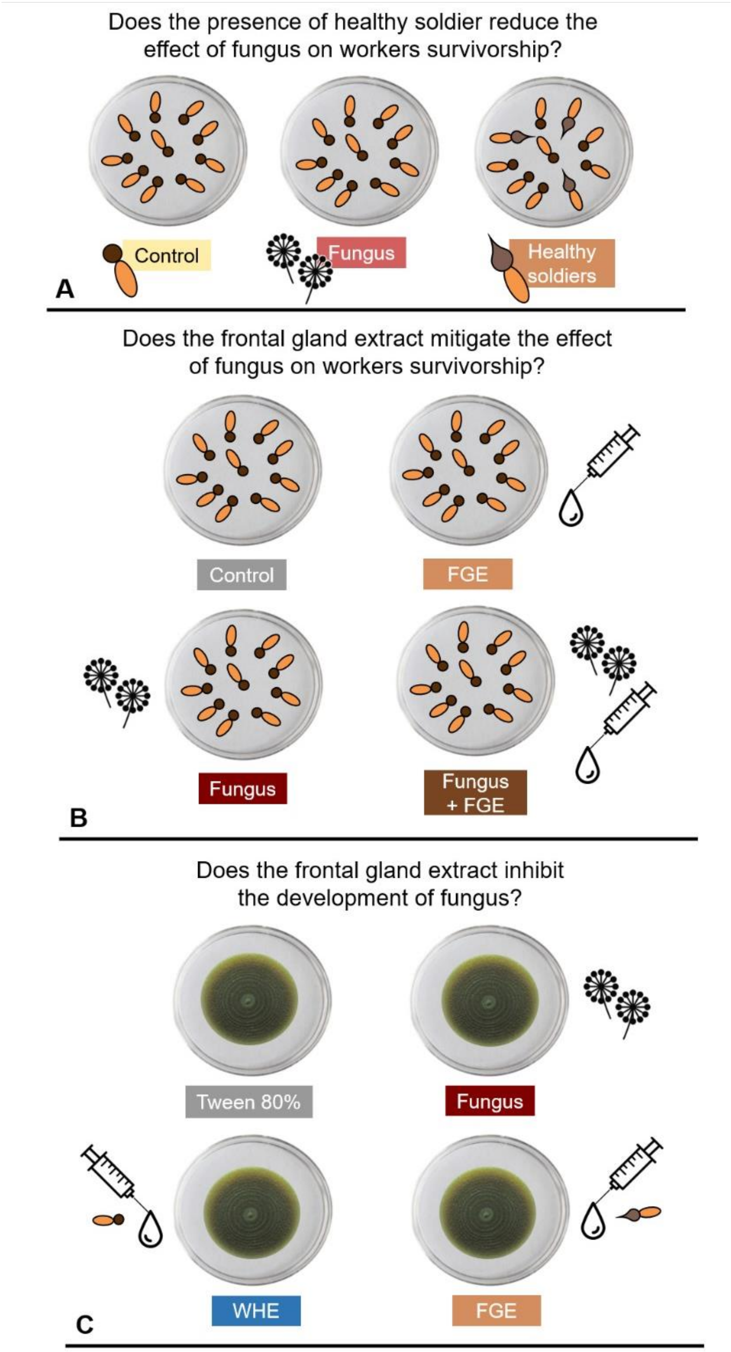

Figure 1. Scheme of experimental design testing the effect of termite's soldiers and its frontal gland extracts on survival of *Nasutitermes corniger* workers and on growth of *Metarhizium anisopliae* fungus. FGE: frontal gland extract. WHE: workers head extracts.

Figure 2. Effect of termite's soldiers and its frontal gland extracts on survival of *Nasutitermes corniger* workers. (A) Positive effect of healthy soldiers on survivorship of *N. corniger* workers. (B) Positive effect of frontal gland extracts of *N. corniger* soldiers on survivorship of *N. corniger* groups composed only with workers. The bars followed by the same letters do not significantly differ by EMMeans post-hoc test. The black dot in the line represents the mean effect sizes. FGE: frontal gland. extract.

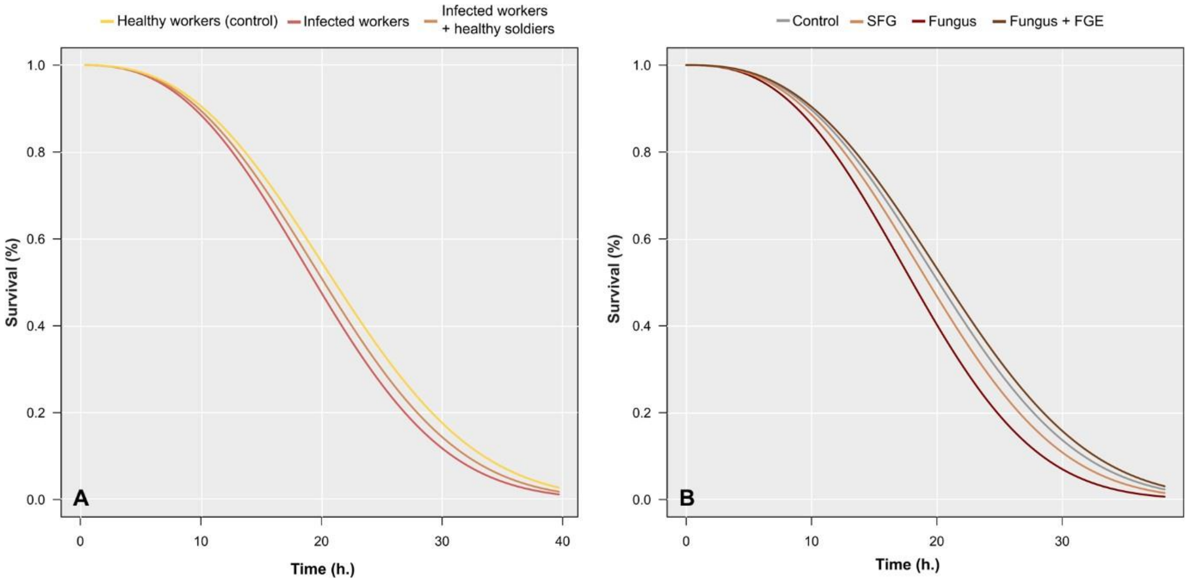

Figure 3. Survival curves of *Nasutitermes corniger* groups showing the dynamic of death over time. (A) Survival curves showing the positive effect of healthy soldiers on survivorship of *N. corniger* workers. (B) Survival curves showing the positive effect of frontal gland extracts of *N. corniger* soldiers on survivorship of *N. corniger* groups. Fungus + FGE: fungus and frontal gland extract.

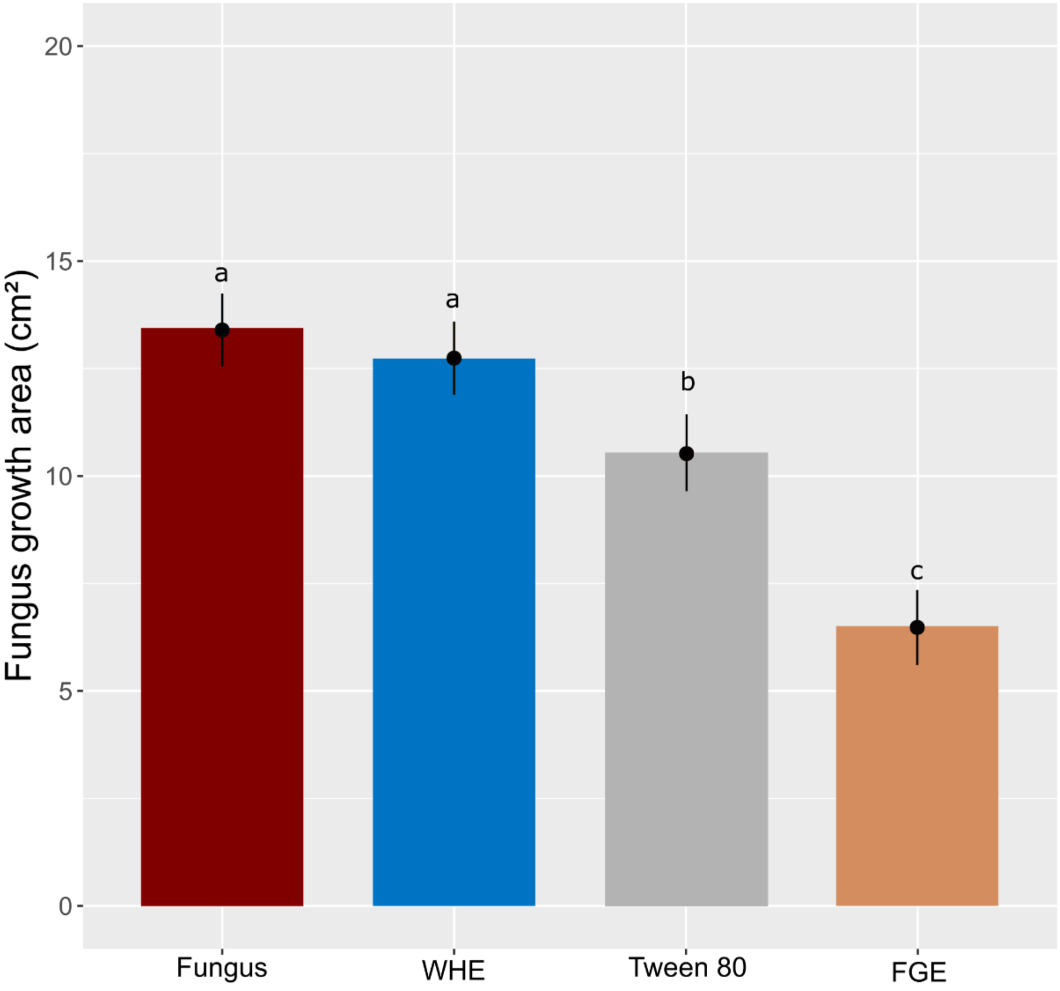

Figure 4. Positive effect of soldier's frontal gland secretion of *Nasutitermes corniger* soldiers in reducing fungal growth after seven days. The bars followed by the same letters do not significantly differ by EMMeans post-hoc test. The black dot in the line represents the mean effect sizes. FGE: frontal gland extract. WHE: workers head extracts.

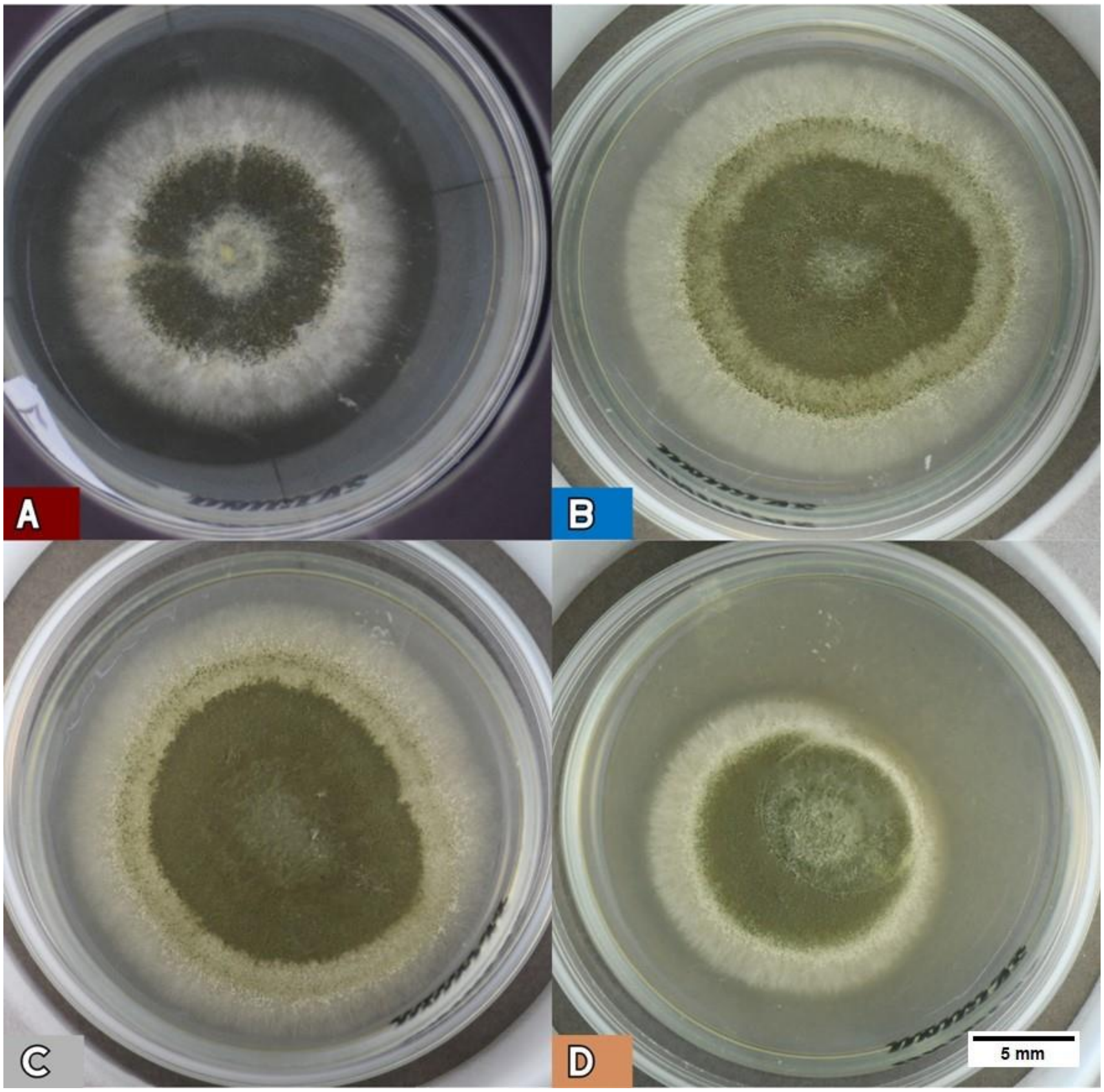

Figure 5. The most representative images of the mycelia (area in cm^2). (A) *Metarhizium anisopliae* fungus suspension. (B) Worker head extract of *N. corniger* and *M. anisopliae* suspension. (C) Tween 80 solution and *M. anisopliae* suspension. (D) Frontal gland extract of *Nasutitermes corniger* and *M. anisopliae* suspension. Scale: 5 mm.

CHAPTER 4

FINAL CONSIDERATIONS

In general, the results obtained in this thesis suggest that mixed groups enhance possible mechanisms of social immunity in the nasute termite *N. corniger*. This seems to indicate that presence of workers and soldiers optimizes defense against pathogenic agents, especially when proportion of soldiers reflects that observed in the natural colonies.

Although eusociality presents a series of benefits, life in high density and with a high degree of kinship can poses an important risk: in the presence of pathogens, most individuals can become infected, leading to the death of the colony. However, eusocial insects present a range of collective mechanisms (called as "*social immunity*"), to overcome the costs imposed by entomopathogens. In *N. corniger,* these mechanisms can play a crucial role in controlling fungal spread and include behaviors such as grooming, trophallaxis, vibration and antennation (Chapter 2; Ferreira *et al.* 2023). Furthermore, soldiers play a fundamental role in the survival of other individuals after exposure to the fungus *Metarhizium anisopliae,* once they possess an effective chemical apparatus with a functional frontal gland that produces substances with antifungal properties (Chapter 3).

Concluding, the present thesis contributes to new insights into social immunity in termites, as well as in the defense mechanisms involved. We hope that the results presented here help to understanding the role of soldiers and generate further hypotheses on the relationship between colony immunity and termite castes.