TOWSON UNIVERSITY COLLEGE OF GRADUATE STUDIES AND RESEARCH

EXTERNAL SECRETIONS AND ANTIFUNGAL DEFENSE IN SUBTERRANEAN TERMITES

by

CASEY HAMILTON

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Department of Biological Sciences

Towson University

Towson, Maryland 21252

Advisor: Dr. Mark S. Bulmer

Committee Members: Dr. Gail Gasparich and Dr. Michelle Snyder

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Casey Hamilton entitled "External secretions and antifungal defense in subterranean termites" has been approved by the thesis committee as satisfactorily completing the thesis requirements for the degree of Master of Science in Biology.

Committee Chair: Mark S. Bulmer

Mal Sch Date: 5/6/11 ember: Gail Gasparich hul & Stappenick Date: 5/10/11

Committee Member: Gail Gasparich

Committee Member: Michelle Snyder

Date: <u>5/6//</u> W/ G

Associate Dean, College of Graduate Studies and Research

Date: 10 Mar m

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ABSTRACT

Termites face strong pathogenic pressures associated with their social organization and ecological attributes. *Reticulitermes* subterranean termites are exposed to the entomopathogenic fungus *Metarhizium anisopliae*, which is a natural pathogen ubiquitous in the soil that these termites nest and forage in. *M. anisopliae* can effectively evade insect innate immune defenses after penetrating the cuticle. Here, I describe an external antifungal defense system utilized by *Reticulitermes* in which salivary gland antifungal secretions with strong GNBP-associated β -1,3-glucanase activity are spread over the cuticle. Inhibition of β -1,3-glucanase activity decreases antifungal activity and increases mortality in termites exposed to a local strain of *M. anisopliae*. RNA interference shows that GNBP2 and termicin are essential antifungal molecules in these termites, and important components of external defenses. This external defense strategy targets fungal pathogens before cuticular penetration, and could have been important in overcoming the pathogenic pressures associated with group living and nesting in soil.

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Introduction

Termites and other social insects live in colonies that can consist of hundreds to thousands of highly related individuals in constant close contact, often sharing fluids and nutrients through stomodeal and/ or proctodeal trophallaxis. Colonies are divided into castes of one or a few reproductive individuals and workers and soldiers, which care for brood, forage, and construct and defend nests. Termites nest and forage in soil and decaying wood, which are ideal environments for bacterial and fungal growth. All of these characteristics make termites and other social insects especially susceptible to microbial infection and the spread of disease among nestmates. In spite of high pathogenic pressures, termites are incredibly successful evolutionarily and are major components of many terrestrial ecosystems throughout the world (Schmid-Hempel, 1998). The question of how these insects successfully overcome these intense pathogenic pressures is of major interest in evolutionary biology.

Insects lack vertebrate-like adaptive immunity and rely on innate cellular and humoral immune defenses to neutralize invading pathogens. Innate immune defenses are triggered by the recognition of conserved pathogen components by membrane-bound and/ or soluble pattern recognition receptors (PRRs), which then activate cellular responses such as phagocytosis and encapsulation, the prophenoloxidase cascade, and the humoral production of antimicrobial peptides (AMPs) (Royet, 2004). Social insects also have behavioral mechanisms to prevent and control infection. Social immunity ranges from removing pathogens from the cuticle through self and allogrooming to hygienic behaviors such as the incorporation of antimicrobials into nest materials and the removal

1.

of infected individuals from nests (Cremer et al., 2007, Rosengaus et al., 2011, Wilson-Rich et al., 2009). For example, honeybees use "social fever" to increase the temperature inside the nest and kill invading bacteria (Starks et al., 2000). Also, some ants control microbial growth inside nests by incorporating antimicrobial conifer resin into nests (Christe et al., 2003). Leaf-cutting ants rely on antibiotic secretions of symbiotic bacteria to prevent harmful microbial growth in fungus gardens and to defend against infection by pathogenic microbes (Little et al., 2006)

While most viruses and bacteria infect insects via the alimentary tract, entomopathogenic fungi such as *Metarhizium anisopliae* secrete enzymes that allow them to directly enter through the cuticle (Gillespie et al., 2000). After invading the insect's hemocoel, *M. anisopliae* employs strategies to effectively avoid cellular and humoral immune defenses (Vey et al., 2002, Wang and St Leger, 2006). *Nasutitermes* termites secrete Gram-negative bacteria binding proteins (GNBPs) that exhibit β -1,3-glucanase activity externally on the cuticle and in nest materials, possibly targeting fungal pathogens before cuticular penetration. This external defense strategy could be essential for preventing *M. anisopliae* from entering the hemocoel and evading cellular and humoral immune defenses (Bulmer et al., 2009).

Reticulitermes subterranean termites are major decomposers of decaying wood and significantly contribute to the recycling of organic matter and to nitrogen fixation in forest ecosystems (Waller and Curtis, 2003). These termites are also major pests in the Eastern United States, causing considerable damage to wooden structures and incurring high costs associated with control and repair of damaged buildings (Su and Scheffrahn, 2002). Subterranean termites can construct extensive foraging galleries in the soil and in decaying wood, and are constantly exposed to *M. anisopliae*, a natural pathogen of *Reticulitermes* (Zoberi, 1995). The use of *M. anisopliae* as an alternative pesticide strategy has been of particular interest as it specifically targets insects, can withstand exposure to the environment, and has no harmful effects associated with humans or other organisms (Wang and Powell, 2004, Zimmerman, 2007). However, *Reticulitermes* seem to be effective at avoiding infection and preventing the spread of *M. anisopliae* among nestmates due to social behaviors like pathogen avoidance as well as the inactivation of fungal conidia in the soil (Chouvenc and Su, 2010, Chouvenc et al., 2008, Rath, 2000).

Evidence of positive selection in termite GNBPs and antifungal termicin peptides suggests strong pressures placed on antifungal defenses in these insects (Bulmer and Crozier, 2004, Bulmer and Crozier, 2006, Bulmer et al., 2010). An antifungal defense strategy that targets *M. anisopliae* before it invades the hemocoel and evades internal immune defenses could have been critical in overcoming the pathogenic pressures associated with group living and nesting in soil. Here, I investigated *Reticulitermes* external antifungal secretions that are essential in defending against locally isolated strains of *M. anisopliae*. In chapter two, I describe an external antifungal defense system that relies on secreted β -1,3-glucanases and protects against infection by local strains of *M. anisopliae* in the sympatric subterranean termite species *R. flavipes* and *R. virginicus*. In chapter three I present RNA interference experiments in *R. flavipes* that identify GNBP2 and termicin as crucial antifungal molecules that are involved in external defenses against *M. anisopliae*.

2.

Subterranean termite prophylactic secretions and external antifungal defenses

Abstract

Termites exploit environments that make them susceptible to infection and rapid disease transmission. Gram-negative bacteria binding proteins (GNBPs) signal the presence of microbes and in some insects directly damage fungal pathogens with β -1,3-glucanase activity. The subterranean termites *Reticulitermes flavipes* and *R. virginicus* encounter soil entomopathogenic fungi such as *Metarhizium anisopliae*, which can evade host immune responses after penetrating the cuticle. An external defense that prevents invasion of fungal pathogens could be crucial in termites, allowing them to thrive under high pathogenic pressures. We investigated the role of secreted β -1,3-glucanases in *Reticulitermes* defenses against *M. anisopliae*. Our results show that these termites secrete antifungal β -1,3-glucanases on the cuticle, and the specific inhibition of GNBP associated β -1,3-glucanase activity with D- δ -gluconolactone (GDL) reduces this activity and can cause significant increases in mortality after exposure to *M. anisopliae*. Secreted β -1,3-glucanases appear to be essential in preventing infection by breaking down fungi externally.

Keywords: β-1,3-glucanase, Gram-negative bacteria binding protein, social insects, prophylaxis, allogrooming, innate immunity

<u>Abbreviations:</u> PRR, pattern recognition receptor; PAMP, pathogen associated molecular pattern; PGRP, peptidoglycan recognition protein; GNBP, Gram-negative bacteria

binding protein; BGRP, β-1,3-glucan recognition protein; AMP, antimicrobial peptide; GH16, family 16 glycoside hydrolases; GDL, D-δ-gluconolactone.

Introduction

Insects lack a vertebrate-like adaptive immune system and instead rely on physical barriers such as the cuticle, as well as innate cellular and humoral defenses that are activated by the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PRRs include peptidoglycan recognition proteins (PGRPs) that bind peptidoglycans in bacterial cell walls, and Gram-negative bacteria binding proteins (GNBPs) and β -1,3-glucan recognition proteins (BGRPs) that recognize lipopolysaccharides in Gram-negative bacteria outer membranes and β -1,3-glucans in fungal cell walls (Royet, 2004). These PRRs can activate cellular responses, including phagocytosis and encapsulation, as well as pathways leading to the secretion of antimicrobial peptides (AMPs) and reactive oxygen intermediates that are important components of the humoral immune response (Royet, 2004, Strand, 2008). Some PGRPs also exhibit direct bactericidal amidase activity (Mellroth and Steiner, 2006).

GNBPs and BGRPs represent a group of homologous proteins that can activate the prophenoloxidase cascade (Ma and Kanost, 2000, Ochiai and Ashida, 2000, Zhang et al., 2003) as well as the Toll and immunodeficiency (IMD) pathways, leading to the production of AMPs (Kim et al., 2000, Warr et al., 2008). These PRRs share sequence homology with bacterial β -1,3-glucanases, and recent work has shown that these molecules duplicated in the ancestor of mollusks and arthropods, diverging into groups that have maintained or lost amino acids essential for β -1,3-glucanase activity (Bragatto et al., 2010). The enzymatically active GNBPs are family 16 glycoside hydrolases (GH16), and contain two active glutamic acid residues in the catalytic cleft (Juncosa et al., 1994). Although these residues have been replaced in many insect GNBPs that act as PRRs, GNBPs and BGRPs that have maintained the active site and exhibit β -1,3-glucanase activity have been identified in several insects, including termites (Bragatto et al., 2010, Bulmer et al., 2009, Genta et al., 2009, Pauchet et al., 2009).

Termites and other social insects live in densely populated colonies that may have highly related individuals in constant close interactions, often sharing fluids through proctodeal and stomodeal trophallaxis. Subterranean termites also nest and forage in soil and decaying wood and may be inbred (Bulmer et al., 2001, Vargo, 2003, Vargo and Husseneder, 2011), making them especially susceptible to the rapid spread of infection throughout a colony (reviewed by Rosengaus et al., 2011). However, social insects including termites have evolved defenses, ranging from individual innate to social immunity (reviewed by Cremer et al., 2007, Rosengaus et al., 2011), that have allowed them to become evolutionarily successful and ecologically dominant. Social immune defenses can include allogrooming and other hygienic behaviors, as well as the incorporation of antimicrobials in nest materials.

A selective sweep in *termicins* which code for antifungal peptides in the subterranean termites *Reticulitermes flavipes* and *R. virginicus* suggests that these termites face strong selective pressures on their antifungal defenses (Bulmer et al., 2010). This selection may be driven in large part by the entomopathogenic fungus *Metarhizium anisopliae*, a natural pathogen of *R. flavipes* which is ubiquitous in the soil they exploit (Zoberi, 1995). *M. anisopliae* invades insects by penetrating the cuticle (Gillespie et al., 2000) and can then evade host immune defenses by producing a collagenous coat that

prevents hemocyte attachment and masks PAMPs (Wang and St Leger, 2006) and by secreting toxins that interfere with host innate immunity (Vey et al., 2002).

Termite GNBPs also show evidence of a response to selective pressure from pathogens that may include *M. anisopliae* (Bulmer and Crozier, 2006). *GNBPs* duplicated prior to the divergence of termites creating paralogous *GNBP1* and *GNBP2* genes, which have undergone positive selection in a specific lineage associated with a transition to living and foraging in the soil (Bulmer and Crozier, 2006). The isolation and characterization of GNBP2 from the termite *Nasutitermes corniger* showed that these proteins have maintained β -1,3-glucanase activity. The inhibition of GNBP2 β -1,3glucanase activity by D- δ -gluconolactone (GDL), a monosaccharide closely related to glucose, led to increased mortality from infection by *M. anisopliae* and opportunistic pathogens (Bulmer et al., 2009).

Termite GNBPs may play an important role in external antifungal defenses by breaking down fungal conidia in the nest and on the cuticle (Bulmer et al., 2009). Externalized antifungal defenses have been reported from multiple sources in termites, metapleural glands in ants, and also in amphibian skin secretions (Rollins-Smith et al., 2005, Rosengaus et al., 2011, Rosengaus et al., 2004, Schlüns and Crozier, 2009). Termicins and GNBPs are highly expressed in termite salivary glands (Lamberty et al., 2001, Yuki et al., 2008), and may be secreted onto the cuticle during allogrooming and into building materials during nest and foraging gallery construction. We investigated if these molecules are externalized in subterranean termites and are important in frontline defenses that prevent fungal pathogens from penetrating the cuticle.

Materials and Methods

Insect and fungal strain collection and maintenance

Reticulitermes flavipes, R. virginicus, and M. anisopliae were collected from Baltimore County, Maryland, in the spring and summer of 2010. Pieces of wood containing dense aggregations of termites were collected and maintained in the lab in dark plastic containers at room temperature with constant moisture. Termite species were identified using intra- and interspecific agonism assays (Polizzi and Forschler, 1998) and mitochondrial rRNA sequences (Bulmer et al., 2010). Local strains of *M. anisopliae* were isolated from soil in close proximity to (< 30 cm) each termite collection site as described by Hughes et al. (2004). Briefly, Tenebrio molitor larvae were placed in soil samples and monitored daily. Dead larvae were surface sterilized in 70% ethanol and placed on sterile moist filter paper. Conidia were collected from individuals that died of *M. anisopliae* infection, then suspended in 0.1% Tween 80 and stored at 4° C. Strains were confirmed as *M. anisopliae* var. *anisopliae* with partial sequence of IGS and 16S DNA. Stock strains of the mutant $\Delta Mcl1$ and its wild type 2575 were kindly provided by Dr. St. Leger at the Department of Entomology, University of Maryland, College Park, Maryland. *GNBP* amino acid alignment

Amino acid sequences of the catalytic cleft and flanking regions of *N. corniger* (*Ncr*) GNBP1 and 2 (Bulmer et al., 2009) and *Sporaptera frugiperda* (*Sf*) SLam (Bragatto et al., 2010), which have been shown to exhibit β -1,3-glucanase activity, were aligned with sequences for *R. flavipes* (*Rf*) and *R. virginicus* (*Rv*) GNBP1 and 2 (Bulmer et al., 2010). To characterize homology of GNBP1 and 2 within species, a 329-residue region

without the leader peptide was compared using BLAST. (GenBank IDs: *Ncr1*, JF683377; *Ncr2*, JF683378; *Sf*, EF641300; *Rf1*, JF683373; *Rf2*, JF683375; *Rv1*, JF683374; *Rv2*, JF683376).

Termite extracts, cuticular washes, and nest material sampling

For crude extracts, two workers were cold-immobilized on ice and then homogenized in 10 µl of 100 mM sodium acetate (pH 5.0) with QIAshredder columns (QIAGEN). For gutted crude extracts, the hindgut containing the bacterial and protozoan symbiont community was first removed, leaving the foregut and salivary gland reservoirs intact, and then prepared as described for crude extracts. Salivary glands and reservoirs of two termites were extracted and vortexed in 10 μ l of 100 mM sodium acetate (pH 5.0). To standardize for differences between species in cuticular washes, surface area was calculated using Meeh's formula, where surface area = $kW^{2/3}$ (W is mass (g) and k is the species constant which is 12 for termites (Sponsler and Appeal, 1990)). For each colony of termites, the average mass of workers used for cuticular washes was used to calculate surface area, and five workers of *R. flavipes* and seven workers of *R. virginicus* were used for each wash. Cold-immobilized workers were placed in 20.5 µl of 0.1% Tween 80 per cm^2 of surface area, gently agitated for 10 seconds, and 20 µl of wash was extracted. Washes used in antifungal assays were filter sterilized with 0.22 micron Ultrafree filters (Millipore), although later we determined that filter sterilization was not necessary to avoid microbial contamination as the termite cuticles appear to be free of microbes. Crude extract, gutted crude extract, and salivary gland and reservoir contents were kept on ice and used in β -1,3-glucanase activity assays. Cuticular washes were used for β -1,3glucanase activity and antifungal assays.

Nest materials were also prepared for β -1,3-glucanase assays. One hundred mg of foraging gallery material from active laboratory-maintained termite colonies as well as foraging gallery material constructed from 500 mg of soil processed by 100 *R. flavipes* workers for five days were washed with 500 µl of 0.1% Tween 80, then centrifuged three times for 1 minute at 16,000 x g, and the supernatant was concentrated to 20 µl with an Ultracel-10 filter (Amicon). A 3 mm by 3 mm piece of filter paper that had been processed by 12 *R. flavipes* workers for 48 hours was soaked in 15 µl of 0.1% Tween 80 for one minute, and the liquid and paper were then separated in a QIAshredder column. Unprocessed filter paper and sterile soil soaked in 0.1% Tween 80 were used as controls. β -1,3-glucanase activity assays

β-1,3-glucanase activity was detected using a gel electrophoresis assay (Kalix and Buchenauer, 1995). Briefly, samples were run on polyacrylamide gels containing Carboxymethyl Curdlan Remazol Brilliant Blue (Loewe Biochemica) and incubated in 100 mM sodium acetate (pH 5.0, optimal pH) for 24 hours. β-1,3-glucanase activity results in the digestion of curdlan (a β-1,3-glucan polymer) and produces distinct clearing zones (bands) in the gel. Crude extract, gutted crude extract, salivary gland and reservoir contents, cuticular washes, and nest material preparations were all analyzed for β-1,3glucanase activity. To characterize the effect of enzyme inhibitors on β-1,3-glucanase activity of cuticular washes, three samples from pooled washes of *R. flavipes* and *R. virginicus* were run on a gel which was then sectioned into separate gel slices and incubated in 100 mM sodium acetate (pH 5.0), 10 mg ml⁻¹ laminarin (a polymer of β-1,3glucans with β-1,6-linkages) (Sigma-Aldrich) / 100 mM sodium acetate (pH 5.0).

To characterize the antifungal activity of termite cuticular washes, in vitro antifungal assays were adapted from Bulmer et al. (2009). Antifungal activity against a local strain of *M. anisopliae* was measured as a decrease in colony forming units (CFUs) relative to controls. Cuticular washes were incubated with 10 μ l of 10⁴ conidia ml⁻¹ in a total volume of 40 µl (50 µg ml⁻¹ ampicillin). Controls included 20 µl of 0.1% Tween 80 in place of cuticular washes. After 24 hours of incubation, samples were plated on potato dextrose agar plates (60 mm) supplemented with 50 µg ml⁻¹ ampicillin and kept at 25° C for four days, followed by CFU counts. Eight controls and eight replicates from three colonies of each species were used to characterize variation among colonies and differences in antifungal activity between species. To determine if the secreted β -1,3glucanase activity associated with GNBPs is essential for external antifungal defenses, GDL was as used as a specific inhibitor. Antifungal assays were performed as described along with separate control and cuticular wash incubations containing 100 mM GDL. Cuticular washes incubated with 10 mg ml⁻¹ laminarin as a competitive inhibitor of β -1,3glucanases were also tested for antifungal activity.

Survival analyses

To characterize the role of GNBP β -1,3-glucanase activity in antifungal defenses of *Reticulitermes* against *M. anisopliae, in vivo* survivorship experiments were performed. Workers from four colonies of *R. flavipes* (n = 572) and two colonies of *R. virginicus* (n = 192) were divided into control and GDL treatments prior to fungal challenge by placing two groups of 24 workers in dishes (60 mm) with sterile filter paper (Whatman 5) moistened with 300 µl of 100 mM sodium acetate (pH 5.0) (NaOAc treatment) or 100 mM GDL / 100 mM sodium acetate (ph 5.0) (GDL treatment) for 24 hours. Workers from NaOAc and GDL treatments were then divided into control or challenge groups. Control groups were placed on filter paper moistened with 300 μ l of sterile 0.1% Tween 80, while challenge groups were placed on filter paper moistened with 300 μ l of 10⁷ conidia ml⁻¹ for 24 hours. Each colony was challenged with its local strain of *M. anisopliae*. After 24 hours, termites were divided into groups of 12 and transferred to sterile dishes with water-moistened filter paper, and survival was monitored daily for 21 days. Dead termites were removed, surface-sterilized, and placed on moist sterile filter paper to confirm infection by *M. anisopliae*.

In a separate experiment, termites were exposed to the mutant $\Delta Mcl1$ strain of M. anisopliae, which does not produce a protective collagenous coat, to determine if M. anisopliae is effective at evading termite immune defenses after penetrating the cuticle and entering the hemocoel. Termites were exposed to 0.1% Tween 80, $\Delta Mcl1$, or 2575 wild type M. anisopliae for 24 hours and survival was monitored as described.

Statistical analyses

CFU counts were analyzed with Shapiro-Wilk tests for normality and Levene's test for homogeneity of variances to confirm assumptions, followed by ANOVA and Tukey's HSD for multiple comparisons. Survival data were analyzed by Cox regression. All analyses were performed in PASW Statistics 17.0.

Results

GNBP catalytic site alignment

Aligned amino acid sequences of the catalytic cleft and flanking regions showed that GNBP2 from *R. flavipes* and *R. virginicus* share 93% sequence identity with *N*.

corniger GNBP2, which has been shown to exhibit β -1,3-glucanase activity (Bulmer et al., 2009). GNBP1 from *R. flavipes* and *R. virginicus* share 80% sequence identity with GNBP2 from *N. corniger*. Both GNBP1 and GNBP2 from *R. flavipes* and *R. virginicus* have maintained the two catalytic glutamic acid residues in the active site that are essential for β -1,3-glucanase activity (Figure 2.1). GNBP1 and GNBP2 share 73% sequence identity in *R. flavipes* and 72% sequence identity in *R. virginicus*.

Ncr2	164	GDWIWPAIWLMPRYNLYGG	WPTSGEIDL	TESRGN	KQLIQNGQ	NIGSELSSS1	LHFGPFWPLNGYERA	230
Rf2	164	F-FS	S		RK	T	Н-	230
Rv2	164	EF-F	S		RE	T	Н-	230
Ncr1	164	LEF	A	S-G2	AN-TSK-I	LKL	QN-	230
Rf1	164	LEF	A	S-G	TN-TLS	SST	YV-AF-HT	230
Rv1	164	LEEEEE	——A—————	S-G	TN-TLS	SST	YF-HT	230
Sf	164	ATT	A	V	RNMFHV	/HTQEAG		230

Figure 2.1. Alignment of amino acids 164-230 that line the catalytic cleft of GNBPs with β -1,3-glucanase activity. The catalytic region is shaded, and the glutamic acids that are critical for catalysis are labeled with asterisks. These residues are substituted with different amino acids in GNBPs that lack catalytic activity. *Ncr*1 and 2 correspond with *N. corniger* GNBP1 and 2; *Rf*1 and 2 correspond with *R. flavipes* GNBP1 and 2; *Rv*1 and 2 correspond with *R. virginicus* GNBP1 and 2; *Sf* corresponds with *Sporaptera frugiperda* β -1,3-glucanase (SLam). Purified *Ncr* GNBP2 and SLam have been shown to exhibit β -1,3-glucanase activity.

<u>β-1,3-glucanase activity</u>

Significant β -1,3-glucanase activity was detected in crude extracts of *R. flavipes* and *R. virginicus* workers (Figure 2.2a). Two prominent clearing zones were visible in both species, with minor bands attributable to the hindgut and not to gutted termite extracts. These are likely digestive β -1,3-glucanases produced by bacterial and protozoan symbionts, which produce a diversity of digestive enzymes (Todaka et al., 2007, Warnecke et al., 2007). Termite salivary glands and reservoirs and cuticular washes also showed significant enzyme activity in both species with band patterns consistent with gutted extracts (Figure 2.2a,b). This β -1,3-glucanase activity was also detected in filter paper that was processed by *R. flavipes* workers (Figure 2.2c), foraging gallery material

constructed from soil by *R. flavipes* workers (Figure 2.2d), and in nest material of both species (not shown). No activity was detected in control filter paper or soil.



Figure 2.2. β -1,3-glucanase activity of *R. flavipes* (*Rf*) and *R. virginicus* (*Rv*) workers visualized on Carboxymethyl Curdlan Remazol Brilliant Blue gels. a) Activity of termite crude extract, gutted extract, and salivary gland and reservoir contents. b) Activity of termite gutted extract and cuticular washes. c) Activity of *R. flavipes* crude extract and filter paper that was processed by 12 workers for 48 hours. d) Activity of *R. flavipes* gutted extract and gallery material constructed from soil by 100 workers for five days. Control filter paper and soil washes showed no activity.

Antifungal activity of cuticular washes

Significant antifungal activity was detected in cuticular washes of *R. flavipes* and *R. virginicus* workers ($F_{2,61} = 253.8$, p < 0.001; Figure 2.3), with significantly lower CFU counts in *R. virginicus* (67% mean CFU reduction) relative to *R. flavipes* (48% mean

CFU reduction) (Tukey's HSD, p < 0.001). Variation among colonies was borderline significant in *R. flavipes* ($F_{2,21} = 3.5$, p = 0.05), and no variation was detected among *R. virginicus* colonies ($F_{2,21} < 0.001$, p = 0.99).



Figure 2.3. Antifungal activity of worker cuticular washes (n = 8 per colony) from three colonies of both *R. flavipes* and *R. virginicus* measured as decreases in CFU counts relative to controls. Controls included incubations with 0.1% Tween 80 in place of cuticular washes. Bars represent mean CFU count (±SD), and different letters indicate significant differences (Tukey's HSD).

Inhibition of β -1,3-glucanase and antifungal activity

 β -1,3-glucanase activity of cuticular washes was inhibited by GDL and laminarin

(Figure 2.4). Laminarin appears to non-specifically interfere with all β -1,3-glucanases as

a general competitive inhibitor, while GDL specifically inhibits the top clearing zone,

which was associated with the specific inhibition of GNBP2 in N. corniger (Bulmer et

al., 2009).



Figure 2.4. Inhibition of β -1,3-glucanase activity of cuticular washes of *R. flavipes (Rf)* and *R. virginicus (Rv)* workers by laminarin and GDL. Gels were incubated in 100 mM sodium acetate (pH 5.0) (NaOAc), 10 mg ml⁻¹ laminarin / 100 mM sodium acetate (pH 5.0) (Laminarin), or 100 mM GDL / 100 mM sodium acetate (pH 5.0) (GDL).

The addition of GDL depleted the antifungal activity of cuticular washes in both species $(F_{5,42} = 31.1, p < 0.001; Figure 2.5)$. While cuticular washes showed significant antifungal activity relative to controls (Tukey's HSD, p < 0.001), washes incubated with GDL showed no antifungal activity, and control incubations containing GDL showed no difference in CFU count relative to controls.



Figure 2.5. Inhibition of cuticular wash antifungal activity by GDL in *R. flavipes* (*Rf*) and *R. virginicus* (*Rv*) (n = 8 per treatment). Controls included 0.1% Tween 80 in place of cuticular washes. GDL incubations contained 100 mM GDL. Species labels designate treatments that included cuticular washes in the incubations. Bars represent mean CFU count (\pm SD), and different letters indicate significant differences (Tukey's HSD).

While washes incubated with laminarin still exhibited significant antifungal activity relative to controls in *R. flavipes* and *R. virginicus* (Tukey's HSD, p < 0.001), laminarin significantly inhibited antifungal activity relative to normal cuticular washes in both species (Tukey's HSD, *R. flavipes* p = 0.002; *R. virginicus* p < 0.001; Figure 2.6).



Figure 2.6. Inhibition of cuticular wash antifungal activity by laminarin in *R. flavipes* (a) and *R. virginicus* (b) (n = 8 per treatment). Controls included 0.1% Tween 80 in place of cuticular washes, and laminarin treatments included 10 mg ml⁻¹ laminarin. Bars represent mean CFU count (\pm SD), and different letters indicate significant differences (Tukey's HSD).

<u>GDL β -1,3-glucanase inhibition and termite survival</u>

Exposure to GDL prior to a fungal challenge with *M. anisopliae* had a significant effect on survival in *R. flavipes* workers (Wald = 116.0, df = 3, p < 0.001; Figure 2.7a). Workers exposed to GDL prior to a fungal challenge exhibited a 2.8 times higher hazard ratio of death relative to NaOAc control challenged termites (Wald = 28.8, df = 1, p <

0.001). Colony effect on survival was not significant (Wald = 3.3, df = 3, p = 0.34). For *R. virginicus* workers, neither treatment (Wald = 2.1, df = 3, p = 0.56) nor colony (Wald = 2.0, df = 1, p = 0.16) had a significant effect on survival (Figure 2.7b).



Figure 2.7. Survival of workers from four colonies of *R. flavipes* (a) (n = 572) and two colonies of *R. virginicus* (b) (n = 192) exposed to 0 mM GDL or 300 mM GDL prior to a challenge with *M. anisopliae* (Ma). Controls were exposed to sterile 0.1% Tween 80 (Tw80).

<u>AMcl1 vs. 2575 wild type and termite survival</u>

Significant differences in survival were detected in *R. flavipes* workers challenged with either $\Delta Mcl1$ or 2575 wild type *M. anisopliae* (Wald = 18.7, df = 1, p < 0.001; Figure 2.8a). Workers exposed to wild type conidia exhibited a 4.5 times higher hazard ratio of death relative to workers exposed to $\Delta Mcl1$ conidia (Wald = 9.9, df = 1, p = 0.002). No differences in survival among treatments were detected in *R. virginicus* (Wald = 1.2, df = 2, p = 0.6; Figure 2.8b).



Figure 2.8. Survival of *R. flavipes* (a) and *R. virginicus* (b) workers (n = 72 per species) challenged with the $\Delta Mcl1$ mutant that does not produce a protective collagenous coat or the 2575 wild type strain of *M. anisopliae*. Controls were exposed to sterile 0.1% Tween 80 (Tw80).

Discussion

The recent recognition that *Nasutitermes* GNBPs have intact β -1,3-glucanase active sites led to an investigation of their external role in antifungal activity in addition to their internal role as PRRs. Purified GNBP2 from *N. corniger* exhibits strong β -1,3glucanase activity that weakens fungal conidia by breaking down β -1,3-glucan, which is a major component of fungal cell walls (Bulmer et al., 2009). The presence of an intact β -1,3-glucanase catalytic site in GNBPs suggests that they also provide an integral role in antifungal defense in *Reticulitermes* subterranean termites, which are distantly related to *Nasutitermes* (Figure 2.1). Our results confirm this prediction; secreted β -1,3glucanases provide protection from a fungal pathogen that subterranean termites naturally encounter when moving through the soil.

The secretion and antifungal activity of β -1,3-glucanases observed in this study indicates that these molecules are important external effector molecules in *Reticulitermes*.

Strong β -1,3-glucanase clearing zones are apparent in the salivary gland and reservoir (Figure 2.2a), which is a known source of termite GNBPs with β -1,3-glucanase activity (Bulmer et al., 2009). These bands are also present on the cuticle and in nest material (Figure 2.2b, c, d). Cuticular washes show significant antifungal activity in both species of subterranean termite against strains of *M. anisopliae* they encounter naturally, suggesting that molecules secreted from the salivary glands and spread over the cuticle are important external defenses against fungal entomopathogens (Figure 2.3). GDL specifically inhibits the clearing zone of β -1,3-glucanase activity that is strongly present in both salivary glands and reservoirs and cuticular washes (Figure 2.4), consistent with the patterns of GNBP2-specific inhibition in *Nasutitermes* (Bulmer et al., 2009). The antifungal activity is completely inhibited by the addition of GDL, which specifically binds the β -1,3-glucanase active site of termite GNBPs, demonstrating that secreted β -1.3-glucanases are essential in the external antifungal activity of the cuticular washes (Figure 2.5). Laminarin also inhibits this antifungal activity, and the weaker effect relative to GDL could be due to concentration or a lower binding affinity and competitive inhibition efficiency (Figure 2.6).

The secretion of antifungal β -1,3-glucanases could provide a selective advantage to subterranean termites, allowing them to preemptively prevent infection from fungi such as *M. anisopliae* that can evade host immune defenses after invading the hemocoel (Vey et al., 2002, Wang and St Leger, 2006). The inhibition of β -1,3-glucanase effector activity with GDL in *R. flavipes* causes increased mortality in termites exposed to their local strain of *M. anisopliae*, demonstrating that this effector activity is a critical component of subterranean termite defenses against natural fungal pathogens (Figure 2.7a). The secretion of a collagenous coat by *M. anisopliae* apparently protects fungal cells from termite immune defenses after invading the hemocoel, as lower mortality is seen in *R. flavipes* workers exposed to a mutant strain ($\Delta Mcl1$) that is unable to produce the collagenous coat (Figure 2.8a). The protective collagenous coat masks β -1,3-glucans of fungal cells, preventing the activation of host immune responses (Wang and St Leger, 2006), and preventing internal GNBPs from signaling the presence of the fungus and/or directly breaking down their cell walls. Because the mutant strain still inflicts moderate mortality, *M. anisopliae* appears to have other means of avoiding termite immune defenses after penetrating the cuticle. Targeting fungal conidia prior to cuticular penetration allows termites to prevent *M. anisopliae* from entering the hemocoel where they can effectively evade immune defenses.

The importance of externalized GNBPs may vary among termite species given that GDL has no effect on the survival of *R. virginicus* workers during a fungal challenge (Figure 2.7b). Alternatively, the variation in mortality between species could be due to experimental differences in the amount of GDL ingested or the number of fungal conidia that attach to each termite, as *R. virginicus* are small relative to *R. flavipes*. Nevertheless both species secrete β -1,3-glucanases with antifungal activity (Figures 2.2-5), and the energetic cost of maintaining external β -1,3-glucanases is likely to be high, which indicates that they are crucial for antifungal defenses in subterranean termites. Workers of *R. virginicus* seem to be less susceptible than *R. flavipes* to fungal infection by both local strains and a generalized strain (2575) of *M. anisopliae* (Figures 2.7,8); this pattern could be due to differences in dosage and exposure efficiencies between species, or it is possible that *R. virginicus* may have redundant or enhanced antifungal defenses. Allogrooming increases in termites exposed to *M. anisopliae* (Rosengaus et al., 1998) and is a mechanism that may facilitate the spread of antifungal secretions over subterranean termite cuticles. In *Reticulitermes*, these secretions include termicins, which are small defensin-like antifungal peptides that may damage cell membranes (Da Silva et al., 2003, Lamberty et al., 2001), as well as GNBPs with β -1,3-glucanase activity that damage cell walls. It is possible that GNBP β -1,3-glucanase activity acts synergistically with termicins, weakening fungal cell walls and allowing termicins access to the cell membrane (Bulmer et al., 2009). Recent results using RNA interference show that termicins are also important in defense against *M. anisopliae* in *R. flavipes* (Hamilton and Bulmer, in preparation). Based on our results, the salivary gland appears to play an analogous role to the metapleural gland in ants, which is an important source of antimicrobial compounds that are spread over the cuticle by many ant species (reviewed by Schlüns and Crozier, 2009), and has been shown to be important in leaf-cutting ant defenses against *M. anisopliae* (Poulsen et al., 2002).

Though distantly related, ants and termites exhibit striking life-history similarities that may place common selective pressures on immune defenses, and the evolution of external protection may have been critical in overcoming intense pathogenic pressures during the evolution of sociality in these insects. The externalization of β -1,3-glucanases on termite cuticles appears to be part of an essential frontline defense strategy against fungal infections, and may have played an important role in the evolution of social behaviors in termites (Bulmer et al., in preparation). Modeling of immunity and hygienic behaviors in social insects has suggested that allogrooming was critical in early stages of social evolution (Fefferman et al., 2007), and combined with the externalization of

antimicrobials may have been important in overcoming the pressures associated with group living in insects. *Reticulitermes flavipes* can inactivate *M. anisopliae* conidia in their environment, possibly through ingestion (Chouvenc et al., 2008, Chouvenc et al., 2009) or the externalization of antifungal secretions described here. These proteins are constitutively expressed and maintained on the cuticle and in nest materials and appear to be important antifungal effector molecules that provide prophylactic measures against *M. anisopliae*. Because subterranean termites appear to use β -1,3-glucanases preemptively to prevent cuticular penetration and infection by *M. anisopliae* and these enzymes can readily be inhibited, they may represent a potential Achilles' heel in a sophisticated defensive strategy.

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3.

Molecular antifungal defenses in subterranean termites: RNA interference reveals *in vivo* roles of termicins and GNBPs against a naturally encountered pathogen

Abstract

Subterranean termites face strong pathogenic pressures from the ubiquitous soil fungus *Metarhizium anisopliae*, and rely on innate humoral and cellular, as well as behavioral immune defenses for protection. *Reticulitermes* termites secrete antifungal enzymes that exhibit strong β -1,3-glucanase activity associated with Gram-negative bacteria binding proteins (GNBPs), which prevent *M. anisopliae* from invading the hemocoel where it can evade immune responses. Molecular evolutionary studies of *Reticulitermes* termicins, which code for defensin-like antifungal peptides, suggest that these proteins may be important effector molecules in antifungal defenses. In this study we show that the RNAi knockdown of termicin and GNBP2 expression via the ingestion of dsRNA significantly increases mortality in termites exposed to a naturally encountered strain of *M. anisopliae*. Termicin and GNBP2 knockdown also decrease external cuticular antifungal activity, indicating a direct role for these proteins in an external antifungal defense strategy that depends on the active dissemination of antifungal secretions among nestmates.

<u>Keywords:</u> Gram-negative bacteria binding protein, termicin, defensin, social insects, Reticulitermes flavipes, Metarhizium anisopliae

<u>Abbreviations:</u> PAMP, pathogen associated molecular pattern; PRR, pattern recognition receptor; Imd, immune deficiency pathway; AMP, antimicrobial peptide; GNBP, Gram-

negative bacteria binding protein; BGRP, β -1,3-glucan recognition protein; RNAi, RNA interference

Introduction

Termites and other social insects face strong pathogenic pressures as they nest and forage in soil and decaying wood. Their social organization depends on sharing nutrients, symbionts, and pheromones, which increases the risk of transmitting disease throughout a colony. Termites and other social insects have evolved a range of defenses that have allowed them to become incredibly successful, ranging from physiological and biochemical individual innate immunity to behavioral and social immunity (reviewed by Cremer et al., 2007, Rosengaus et al., 2011). Innate immunity in termites includes cellular and humoral defenses that are activated by the recognition of pathogen associated molecular patterns (PAMPs), which are conserved structural features of pathogens, including peptidoglycans in bacterial cell walls, lipopolysaccharide (LPS) in Gramnegative bacteria outer membranes, and β -1,3-glucans in fungal cell walls. Pattern recognition receptors (PRRs) bind PAMPs and trigger the Toll and/ or immune deficiency (Imd) pathways, ultimately leading to the secretion of antimicrobial peptides (AMPs) into the hemocoel (Royet, 2004). AMPs are secreted from the fat body and hemocytes upon infection and are a major component of humoral immune defenses in insects (Bulet and Stöcklin, 2005). PRRs can also activate cellular immune defenses such as encapsulation and phagocytosis in response to PAMP recognition and binding (Strand, 2008).

Termicins are a class of AMPs in termites with strong antifungal activity that are constitutively expressed in termite salivary glands and hemocytes, where they may be released into the hemocoel upon infection (Lamberty et al., 2001). They possess an $\alpha\beta$ motif stabilized by three disulfide bridges, similar to insect defensins, and may target the cell membrane of fungi and some Gram-positive bacteria (Da Silva et al., 2003). Gramnegative bacteria binding proteins (GNBPs) are a class of PRRs that can recognize LPS and β -1,3-glucans, and have been shown to be involved in activating the Toll and Imd pathways (Kim et al., 2000, Warr et al., 2008) and the prophenoloxidase cascade in insects (Ma and Kanost, 2000). GNBPs and β -1,3-glucan recognition proteins (BGRPs) share sequence homology with bacterial β -1,3-glucanases, and have diverged into groups of proteins that have maintained or lost active amino acids required for β -1,3-glucan hydrolysis (Bragatto et al., 2010). GNBPs duplicated prior to the divergence of termites, and both GNBP1 and GNBP2 have maintained active β -1,3-glucanase sites. Purified termite GNBP2 has been shown to exhibit direct antifungal effector activity by breaking down β-1,3-glucans in fungal cell walls (Bulmer et al., 2009). Termite GNBPs also serve as PRRs that trigger an innate immune response in addition to their role as direct antifungal effector molecules. Termite GNBPs are expressed in both the salivary glands and the surface of granular hemocytes, and the presence of glycosylphosphatidylinisotol (GPI) anchors suggests both membrane-bound and soluble forms (Bulmer et al., 2009).

Evidence of positive selection has been observed in termicins and GNBPs, suggesting that there has been a molecular arms race between termites and fungal pathogens (Bulmer and Crozier, 2004, Bulmer and Crozier, 2006, Bulmer et al., 2010). Selection in termite GNBPs belonging to *Nasutitermes* species may also have been driven by a shift in microhabitat from epigeal nesting and grass diets to subterranean nesting and diets containing decaying cellulosic material (Bulmer and Crozier, 2006). *Reticulitermes* subterranean termites nest and forage in soil and are constantly exposed to the entomopathogenic fungus *Metarhizium anisopliae*, which is a natural pathogen of these termites (Zoberi, 1995). This fungus can effectively evade insect immune defenses after penetrating the cuticle (Vey et al., 2002, Wang and St Leger, 2006), and may be the main source of pressures on termite antifungal defenses, as it is ubiquitous in the soil they exploit. *Reticulitermes* have evolved an external defense strategy in which antifungal β -1,3-glucanases and possibly other antifungal molecules secreted from the salivary gland are externalized in nest materials and spread over the cuticle during self- and allogrooming. The inhibition of GNBP associated effector activity effectively reduces this external antifungal activity and increases termite mortality after exposure to a local strain of *M. anisopliae* (Hamilton et al., submitted). The externalization of antifungal secretions preventing fungi from penetrating the cuticle could have been important in overcoming intense pathogenic pressures associated with group living during the evolution of sociality in insects.

RNA interference (RNAi) is a powerful tool in functional genomics that has been used to characterize many genes in insects (Bellés, 2010). Immune genes may be particularly susceptible to knockdown via RNAi (Terenius et al., 2011), and this technique has been used to characterize immune genes involved in pattern recognition, Toll and Imd signaling pathways, and humoral immune defenses in insects (Blandin et al., 2002, Eleftherianos et al., 2007, Eleftherianos et al., 2006, Foley and O'Farrell, 2004, Goto et al., 2003, Pili-Floury et al., 2004, Schlüns and Crozier, 2009). RNAi has successfully been used to characterize genes in termites through injection of dsRNA (Korb et al., 2009, Zhou et al., 2006) and also through dsRNA feeding (Zhou et al., 2008). Efficient gene knockdown via the ingestion of specific dsRNA has been used in a variety of insects (Aroujo et al., 2006, Baum et al., 2007, Bautista et al., 2009, Griebler et al., 2008, Kumar et al., 2009, Turner et al., 2006, Whyard et al., 2009, Zhou et al., 2008), and is being considered as a potential tool for pest control (Huvenne and Smagghe, 2010).

Here, we investigated the roles of termicins and GNBPs in antifungal defenses of the subterranean termite *R. flavipes* against local strains of its natural pathogen *M. anisopliae*. We utilized RNAi via the ingestion of specific dsRNA to determine if these proteins are important for *in vivo* antifungal protection against natural pathogens, and if they are directly involved in the external antifungal defense system of *Reticulitermes* subterranean termites.

Materials and Methods

Termite and fungal strain collection and maintenance

R. flavipes and *M. anisopliae* were collected from Baltimore County, Maryland in the spring of 2010. Pieces of wood containing dense aggregations of termites were collected and stored in dark plastic containers in the laboratory under room temperature and constant moisture. Species were identified using intra- and inter-specific agonism assays (Polizzi and Forschler, 1998) and mitochondrial rRNA sequence (Bulmer et al., 2010). Strains of *M. anisopliae* in close proximity (< 30 cm) to each termite collection site were collected as described by Hamilton et al. (submitted) using a baiting technique that utilizes the susceptibility of *Tenebrio molitor* larva when maintained in soil samples to infection by *M. anisopliae* (Hughes et al., 2004). *M. anisopliae* conidia were suspended in 0.1% Tween 80 and stored at 4° C. Strains were identified as *M. anisopliae* var. *anisopliae* with partial sequence of IGS and 16S DNA.

dsRNA preparation

Ten *R. flavipes* workers were cold-immobilized and gently crushed in 100 µl of RNAlater (QIAGEN), kept at 4° C for 24 hours, then stored at -20° C. mRNA was isolated with an illustra QuickPrep Micro mRNA Purification kit (GE Healthcare). cDNA was prepared with the SuperScript III One-Step RT-PCR System (Invitrogen) using primers with T7 polymerase promoter sequences (TAA TAC GAC TCA CTA TAG GG) at the 5' end (termicin: 228 basepairs, forward: AAC AAG TCA ACG GCT ACT ACA ATG, reverse: AAC CAA CTT CCT CTC AAA TGA CTA; GNBP1: 197 basepairs, forward: ATC TGC AGC GGA GAT CTC AT, reverse: AAC CTC TCG CCG TAT TTG TCT; GNBP2: 443 basepairs forward: ACG AGG AAT TCG ACA CTT TCG; reverse: TAT CTC ACC AGA AGA AGG CCA). Preliminary in vivo experiments suggested that ingestion of dsRNA might prime termite immune systems; therefore two separate nonspecific controls were used to ensure that any *in vivo* effect of dsRNA ingestion was not due to the specific sequence of a single non-targeting dsRNA. Two sections of the pGEM-T Easy Vector (Promega) with no known sequence homology to insects (BLAST comparison with NCBI database) were amplified with primers containing T7 promoter sequences to produce templates for non-specific dsRNA synthesis (pGEM-T 1: 435 basepairs, forward: AGG CAC CTA TCT CAG CGA TCT, reverse: TTC TGA CAA CGA TCG GAG GA; pGEM-T 2: 394 basepairs, forward: ACG CTC AAG TCA GAG GTG G, reverse: AGT GTA GCC GTA GTT AGG CCA). PCR and RT-PCR conditions were as follows: pGEM-T non-specific controls, 2 min at 94° C, 40 cycles of 30 sec at

94° C, 30 sec at 55° C, 30 sec at 72° C; *termicin*, *GNBP1*, *GNBP2*, 20 min at 50° C, 2 min at 94° C, 40 cycles of 20 sec at 94° C, 30 sec at 50° C, 45 sec at 72° C.

cDNA was purified with a QIAquick PCR purification kit (QIAGEN) and used as template for *in vitro* transcription with the MEGAscript RNAi Kit (Ambion). Transcriptions were run overnight according to the manufacturers instructions, and purified dsRNA concentrations were determined using a NanoDrop 2000 (Thermo Scientific). Short interfering RNAs (siRNAs) are effective at mediating RNAi in *R. flavipes* via ingestion (Zhou et al., 2008), and ingestion of siRNAs in insects may be more efficient at mediating knockdown effects than long dsRNA (Kumar et al., 2009, Terenius et al., 2011). Therefore, long dsRNA was digested with RNase III (Ambion) according to the manufacturer's instructions to produce short interfering RNAs (siRNAs), which were precipitated in four volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2) and resuspended in nuclease free water to a final a concentration of 0.15 µg ml⁻¹. 20 µl aliquots were stored at -20° C.

dsRNA feeding and semi-quantitative RT-PCR

RNAi treatment was adapted from Zhou et al. (2008). Twelve workers were placed in dishes (60 mm) with 15 mm paper towel discs soaked in 20 µl of water containing: no dsRNA (naïve), 3 µg non-specific dsRNA (pGEM-T 1 or 2), 3 µg of *termicin*-specific dsRNA, 3 µg of *GNBP1*-specific dsRNA, or 3 µg of *GNBP2*-specific dsRNA. Termites were allowed to ingest treated paper for 48 hours. Dosage was based on initial experiments that showed effects on mRNA levels and *in vivo* survival after treatment with 3 µg of dsRNA for 48 hours.

To characterize the effect of specific dsRNA on mRNA levels, semi-quantitative RT-PCR was performed using a procedure adapted from Bragatto et al. (2010). Individual termites from each treatment were cold-immobilized and gently crushed in 20 μl of RNAlater at 4° C for 24 hours, followed by mRNA purification and elution in 50 μl. Four µl of mRNA was used as template in 25 µl RT-PCR reactions with the following primers: termicin, forward: GAA CAA GTC AAC GGC TAC TAC AAT G, reverse: CGA ACT TCC TGC CAA ATG ACT A; GNBP1, forward: ATC TGC AGC GGA GAT CTC AT, reverse: AAC CTC TCG CCG TAT TTG TCT; GNBP2, forward: ACG AGG AAT TCG ACA CTT TCG, reverse: TAT CTC ACC AGA AGA AGG CCA. β-actin expression was used as a control (forward: CAA TAG TGA TGA CCT GGC CGT, reverse: AGA GGG AAA TCG TGC GTG AC). Initial RT-PCR reactions were performed to identify appropriate cycle numbers for each set of primers so that the reaction was interrupted during the log phase of amplification. Conditions were as follows: termicin, 5 min at 50° C, 2 min at 94° C, 34 cycles of 15 sec at 94° C, 30 sec at 50° C, 30 sec at 72 ° C; GNBP1, 5 min at 55° C, 2 min at 94° C, 37 cycles of 15 sec at 94° C, 30 sec at 60° C, 30 sec at 72° C; GNBP2, 5 min at 50° C, 2 min at 94° C, 37 cycles of 15 sec at 94° C, 30 sec at 50° C, 30 sec at 72° C; β-actin, 5 min at 50° C, 2 min at 94° C, 34 cycles of 15 sec at 94° C, 20 sec at 50° C, 45 sec at 72° C. These primers did not amplify PCR products (40 cycles) from dsRNA that had been processed with the Quickprep Micro mRNA kit, which verified that ingested dsRNA was not amplified with this procedure. Two µl of PCR product were run on 1.4% agarose gels prestained with SYBR Safe DNA stain (Invitrogen).

<u>Survival analysis</u>

To test the effect of ingestion of *termicin*, *GNBP1*, and *GNBP2* dsRNA on *in vivo* protection against fungal infection, termites were challenged with a specific strain of *M. anisopliae* isolated from soil alongside their foraging galleries. Termites from each dsRNA feeding treatment were divided into control (Tween 80) or challenge (local strain of *M. anisopliae*) groups; 24 termites were placed in dishes (60 mm) lined with filter paper (Whatman 5) inoculated with either 300 µl of sterile 0.1% Tween 80 or 10^7 conidia ml⁻¹ of *M. anisopliae*. After 24 hours termites were divided into groups of 12, placed in dishes with filter paper moistened with sterile water, and survival was tracked for 21 days. Dead individuals were removed, surface-sterilized in 70% ethanol, and placed on moist sterile filter paper to confirm infection by *M. anisopliae* (green muscardine appearance after 3-5 days). Survival of workers from three colonies (n = 864) was analyzed with a Cox proportional regression.

Cuticular wash antifungal activity

To determine the effect of RNAi treatment on the external antifungal activity on termite cuticles, *in vitro* antifungal activity assays were performed as described by Hamilton et al. (submitted). Briefly, five workers were cold-immobilized, placed in 30 μ l of 0.1% Tween 80, and gently agitated for 10 seconds. The liquid was then filter sterilized (Amicon 0.22 micron Ultrafree filters), and 20 μ l of the filtrate was incubated at 25 ° C with 10 μ l of 10⁴ conidia ml⁻¹ of a local strain of *M. anisopliae* (approximately 100 conidia), and 10 μ l of 200 μ g ml⁻¹ ampicillin (50 μ g ml⁻¹ final concentration) for 24 hours. Incubations were plated on potato dextrose agar plates (60 mm) supplemented with 50 μ g ml⁻¹ ampicillin and stored at 25° C for 4 days, followed by counts of colony forming units (CFUs). Control incubations included 20 μ l of filter-sterilized 0.1% Tween

80 in place of cuticular washes. Eight replicate washes from each dsRNA feeding treatment and eight controls were prepared. CFU counts were analyzed with a Shapiro-Wilk test and Levene's test to confirm assumptions, followed by ANOVA and planned contrasts for pairwise comparisons to determine differences between non-specific dsRNA treatment and knockdown treatments. All analyses were performed using PASW Statistics 17.0.

Results

Gene knockdown

Semi-quantitative RT-PCR showed decreased mRNA levels in termites fed specific dsRNA relative to non-specific dsRNA (pGEM-T) (Figure 3.1). Expression of *termicin* was decreased relative to controls, and non-specific dsRNA ingestion appears to upregulate *termicin* expression relative to naïve termites (Figure 3.1a). The knockdown of *GNBP1* expression was not as substantial relative to expression levels in naïve and non-specific dsRNA treatments (Figure 3.1b). *GNBP2* mRNA levels were also decreased in termites fed specific dsRNA relative to non-specific controls (Figure 3.1c). Decreased mRNA levels in specific dsRNA treatments relative to nonspecific dsRNA controls were reproduced in three separate feeding experiments.



Figure 3.1. Messenger RNA expression levels of termites fed no dsRNA (naïve), nonspecific dsRNA (pGEM-T), or gene-specific dsRNA (*termicin, GNBP1*, and *GNBP2*). βactin expression was used as a control for each sample. a) *termicin* mRNA levels; b) *GNBP1* mRNA levels; c) *GNBP2* mRNA levels.

<u>Survival</u>

Significant differences in survival were detected among treatments (Wald = 247.6, df = 11, p < 0.001; Figure 3.2) and among colonies (Wald = 9.7, df = 1, p =0.002). Relative to termites fed non-specific dsRNA (pGEM-T1), termicin and GNBP2 knockdown treatments showed 7.6 (Wald = 54.3, df = 1, p < 0.001) and 2.0 (Wald = 5.4, df = 1, p = 0.02) times higher hazard ratios of death when exposed to local strains of M. anisopliae. Challenged naïve termites fed no dsRNA also showed a 3.0 times higher hazard ratio of death relative to termites fed non-specific dsRNA (Wald = 14.7, df = 1, p < 0.001). Termites fed GNBP1 dsRNA showed no significant differences in survival during a fungal challenge relative to termites fed non-specific dsRNA (Wald = 0.1, df = 1, p = 0.717). Both non-specific dsRNA treatments (pGEM-T 1 and 2) showed similar patterns of survival after exposure to *M. anisopliae* (Wald = -0.2, df = 1, p = 0.674). Because both non-specific dsRNA treatments showed enhanced survival relative to naïve termites, immune priming is likely a general response to ingestion of dsRNA. All three colonies showed the lowest survival in termicin knockdown treatments, while GNBP2 knockdown caused significant mortality during a fungal challenge relative to non-specific controls in one colony.



Figure 3.2. Survival patterns after RNAi treatment of termites from three colonies (n = 864) exposed to 0.1% Tween 80 (Tw80) or a strain of *M. anisopliae* (Ma) isolated from soil adjacent to each termite collection site. Prior to a fungal challenge, termites were fed filter paper containing no dsRNA (naïve: N), non-specific dsRNA (pGEM-T 1 and 2: P1 and P2), *termicin*-specific dsRNA (T), *GNBP1*-specific dsRNA (G1), or *GNBP2*-specific dsRNA (G2).

Cuticular antifungal activity

Significant differences were detected in antifungal activity ($F_{5,42} = 39.5$, p < 0.001; Figure 3.3). All treatments showed significant antifungal activity relative to control incubations without cuticular washes. However, termicin (53% mean CFU reduction) and GNBP2 (53% mean CFU reduction) knockdown treatment exhibited significantly less antifungal activity relative to non-specific (pGEM-T) controls (69% mean CFU reduction) (termicin, t = 2.7, df = 42, p = 0.009; GNBP2, t = 2.8, df = 42, p = 0.007). Naïve (58% mean CFU reduction) and GNBP1 (74% mean CFU reduction) treatments showed no significant difference relative to non-specific controls (Naïve, t = 1.9, df = 42, p = 0.071; GNBP1, t = -0.8, df = 42, p = 0.437).



Figure 3.3. Antifungal activity of cuticular washes measured as decreases in mean *M. anisopliae* colony forming unit (CFU) counts (\pm SD). Controls included 0.1% Tween 80 in place of cuticular washes. Stars indicate significant pairwise differences relative to nonspecific dsRNA (pGEM-T) incubations from planned contrasts.

Discussion

The suppression of both termicin and GNBP2 with RNAi shows that they are crucial in preventing infection from strains of *M. anisopliae* that *R. flavipes* encounter while foraging for food and constructing nests (Figure 3.2). *Reticulitermes* spread antifungal secretions on their cuticles and incorporate them into nest materials, providing an external defense system that limits the opportunity for pathogens such as *M. anisopliae* to attach and germinate through the cuticle where it can effectively evade insect immune defenses (Hamilton et al., submitted). Both termicins and GNBPs are highly expressed in termite salivary glands and their secretion from this gland together with dissemination by allogrooming appears to be critical for their effector role in external antifungal defense (Bulmer et al., 2009, Hamilton et al., submitted, Lamberty et al., 2001). Termicins and GNBPs may act together in order to destroy fungal cell walls; GNBPs degrade cell wall β-1,3-glucans, allowing termicins to access and fatally interfere

with fungal cell membranes (Bulmer et al., 2009, Rosengaus et al., 2011). Termites therefore appear to have co-opted innate immune proteins for external defense that are usually produced internally by hemocytes and the fat body. This strategy appears to successfully counteract an apparent vulnerability to fungal pathogens that is attributable to the social organization and microhabitat of subterranean termite colonies.

Although significant antifungal activity is apparent on the cuticles of termicin and GNBP2 knockdown termites, the strength of external antifungal activity decreases upon termicin or GNBP2 knockdown (Figure 3.3). This is consistent with previous results showing a decrease in external antifungal activity with the specific inhibition of GNBP associated β -1,3-glucanase activity (Hamilton et al., submitted). Both termicins and GNBPs are constitutively expressed in the salivary gland and stored in reservoirs. These proteins are also constitutively secreted and maintained on termite cuticles, and high levels may persist during RNAi knockdown. Zhou et al. (2008) also observed significant decreases in protein levels 48 hours after Hex-1 dsRNA ingestion and corresponding decreases in endoglucanase activity after Cell-1 knockdown. However, RNAi via feeding in *Reticulitermes* does not completely silence expression (Zhou et al., 2008), and salivary gland secretions on the cuticle and in reservoirs likely provide significant antifungal effector activity even during RNAi treatment.

Termicin and GNBP2 may also be crucial effector molecules for attacking fungal cells that successfully penetrate the cuticle. As well as acting as direct antifungal effectors through β -1,3-glucanase activity, termite GNBPs can act as PRRs, activating immune defenses (Bulmer et al., 2009). Decreased levels of termicin and GNBP2 in the hemolymph would weaken the humoral immune defense system, and decreased GNBP2

levels on hemocytes and in the hemolymph would also reduce the activation of cellular responses mediated by the recognition of fungal PAMPs. Compromised cellular and humoral immune defenses could allow *M. anisopliae* cells that successfully penetrate external defenses to infect termites and cause disease.

The ingestion of GNBP1-specific dsRNA does not affect survival during a fungal challenge or decrease the activity of antifungal secretions. It is possible that GNBP1, which shares 73% sequences identity with GNBP2 and has maintained a β -1,3-glucanase active site (Hamilton et al., submitted), may not be involved in antifungal defenses in termites. However, knockdown of GNBP1 may not have been sufficient for *in vivo* or *in vitro* effects as decreases in mRNA levels were not as substantial as those observed for termicin and GNBP2 (Figure 3.1). In addition, the population of siRNAs targeting GNBP1 mRNA could be less efficient at mediating RNAi, as the template used for dsRNA synthesis was short relative to termicin and GNBP2 templates. Furthermore, the efficacy of RNAi can vary greatly depending on dose, target mRNA and siRNA stability, mRNA levels and feedback mechanisms, tissue-type, and life-stage (Terenius et al., 2011).

A general upregulation of immune defenses is stimulated by dsRNA, as ingestion of non-specific dsRNA increases survival of termites during a fungal challenge (Figure 3.2). The RNAi pathway evolved as a defense against viruses and transposable elements (Obbard et al., 2009), and dsRNA may elicit a broad innate immune response. Upregulation of immune genes and AMP transcription after injection of non-specific dsRNA has been reported in bees (Schlüns and Crozier, 2007). Viral dsRNA activates the Toll pathway and the production of AMPs in *Drosophila* (Zambon et al., 2005), consistent with the apparent upregulation of termicins in response to non-specific dsRNA. Similarly, Toll-like receptors in mammalian cells recognize viral dsRNA and activate an innate immune response (Alexopoulou et al., 2001).

We previously reported that positive selection resulted in a selective sweep of termicins in *Reticulitermes*, which is a response to strong selective pressures on subterranean termite antifungal defenses (Bulmer et al., 2010). The selective sweep reduced polymorphism and was likely driven by pathogenic pressures placed on these termites by *M. anisopliae*. The strength of this selective sweep appeared to be stronger in *R. flavipes* than *R. virginicus*, a close sympatric relative of *R. flavipes*. In this study, all the R. flavipes colonies examined showed increased mortality upon a fungal challenge after termicin knockdown. R. virginicus, which also secrete antifungal β-1,3-glucanases on the cuticle (Hamilton et al., submitted), did not show evidence of decreased mRNA levels and increased vulnerability to *M. anisopliae* with *termicin* and *GNBP* dsRNA treatments (data not shown). This may be due to differences in dsRNA dosage and ingestion or inherent differences in RNAi mechanisms and siRNA efficacies between species. Alternatively, *R. virginicus* may have termicins and GNBPs that are especially effective against *M. anisopliae* so that even partial knockdown does not affect survivorship, or perhaps R. virginicus has additional systems of protection. However, the experimental differences do not necessarily reflect greater R. flavipes vulnerability to M. anisopliae in a natural context. Both species are likely to have highly effective antifungal resistance against the lower concentrations of *M. anisopliae* naturally encountered in the soil, which range from 10^3 to 10^4 CFUs g⁻¹, and rarely exceed 10^6 CFUs g⁻¹ (Hughes et al., 2004, Roberts and St Leger, 2004). The effect of GNBP2 knockdown on termite

survival varied among *R. flavipes* colonies; this could be due to variation in the efficacy of these proteins among colonies, as polymorphism in *Reticulitermes* GNBP2s is high relative to termicins (Bulmer et al., 2010).

The incorporation of antifungal effector molecules in nest materials may also function as an external recognition system, releasing digested fungal cell wall components that could elicit an immune response and prime the immune defenses of termites that are working in infested regions of their nests or foraging territories (Bulmer et al., 2009, Hamilton et al., submitted). Termicins and GNBPs therefore appear to provide multifaceted protection as internal effectors and receptors and as external effectors and sensors. Their novel role in termite immunity has apparently evolved in response to termites' vulnerability to pathogens due to their sociality, but has also evolved in conjunction with the adaptive behavioral innovations afforded by their sociality.

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4.

Reticulitermes subterranean termites utilize an external antifungal defense system that provides protection from naturally encountered strains of *M. anisopliae*. Antifungal salivary gland secretions are spread over the cuticle and in nest materials, attacking fungal conidia before they can enter the hemocoel. GNBPs in *Reticulitermes* are essential for external antifungal defenses through direct β -1,3-glucanase effector activity, and the inhibition of this activity depletes the efficacy of antifungal secretions and increases susceptibility to fungal infection. Both termicins and GNBP2s are directly involved in and crucial for antifungal defenses in *R. flavipes*. Knockdown of termicin or GNBP2 significantly increases mortality after exposure to local strains of *M. anisopliae* and also decreases the activity of external antifungal secretions.

This defense strategy may have been essential for overcoming the pressures placed on these termites by soil entomopathogens such as *M. anisopliae* during the evolution of sociality in termites. Targeting fungal pathogens prior to cuticular penetration would circumvent fungal mechanisms of evading host immune defenses. The externalization of antifungal secretions via social behaviors was likely critical for overcoming the costs and pressures associated with group living and nesting in soil, where risks of exposure to *M. anisopliae* and the spread of infection among nestmates are especially high.

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