Symbiont-mediated protection against fungal infection in the dampwood termite, *Zootermopsis angusticollis*.

A thesis presented

by

Kelley Frances Schultheis

to
The Department of Biology

In partial fulfillment of the requirements for the degree of
Master of Science

in the field of

Biology

Northeastern University,
Boston, Massachusetts
August, 2009
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ABSTRACT OF THESIS

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Abstract

Termites have a long co-evolutionary history with prokaryotic and eukaryotic gut microbes. Historically, the role of these obligate symbionts has been attributed to the nutritional welfare of the host. We hypothesize, however, that the nature of this mutualistic interaction extends beyond the nutritional benefits to the host and propose that termite gut symbionts enhance the host’s defenses against pathogens. To test this hypothesis, a series of experiments were devised using the primitive dampwood termite Zootermopsis angusticollis and the entomopathogenic fungus Metarhizium anisopliae. In order to examine the role of symbionts in host protection, termites were experimentally defaunated (obligate anaerobic hindgut symbionts removed) with oxygen and compared with control animals in in vivo and in vitro experiments.

First, both defaunated and faunated control termites were exposed to the conidia of M. anisopliae or to a control Tween 80 solution and their survival was tracked for 21 days. In vivo data indicate that normally faunated termites are significantly less susceptible to M. anisopliae infection than their defaunated counterparts. In addition, defaunated termites are more susceptible to opportunistic pathogens, such as Serratia sp., than their faunated nestmates. The hindgut community of Z. angusticollis appears to play an important role in pathogen defense. To further characterize this role, two additional experiments were conducted.

Faunated and defaunated Z. angusticollis individuals were tested for β-1,3 glucanase (βGlu) activity. The gut of Z. angusticollis has several active βGlu’s. We have determined that this βGlu activity is of symbiont-origin and that their presence in the gut may help explain the higher survival of faunated fungal-infected termites compared to defaunated fungal-infected nestmates. We have concluded that these βGlu’s likely play an important role in termite pathogen defense. In addition to the symbiont derived βGlu’s, we have also identified two βGlu’s of potential termite origin. Furthermore, the eastern subterranean termite, Reticulitermes flavipes,
also exhibits symbiont-produced βGlu’s, indicating that this could be a widespread phenomenon in the phylogenetically basal (“lower”) Isoptera.

Lastly, via in vitro experiments, we investigated the ability of termite tissue to inhibit the germination of *M. anisopliae* by incubating conidia with termite extracts. The fungistatic effect of faunated guts, measured by colony forming units (CFUs), tended to be stronger than that of the faunated gutted body, demonstrating that factors in intact guts make it particularly inhospitable to fungi. To test if the fungistatic nature of the gut was due to the presence of symbionts, extracts of faunated and defaunated insects were also incubated with fungal conidia. There were no significant differences in median number of CFUs between conidia incubated with faunated and defaunated termite extracts. One possible explanation for this is that bacterial symbionts not eliminated during oxygenation could be “compensating” by producing more or different antifungal compounds. Blocking the βGlu activity with D-Glucon-1,5 lactone (GDL) in an attempt to “rescue” conidia viability following incubation with termite extracts did not have a consistent effect on *M. anisopliae* germination. This could potentially be connected to incomplete βGlu inhibition by the GDL. The main contribution of this research lies in establishing a novel disease defense role for the mutualistic association between termites and their hindgut symbionts, which in turn contributes to a better understanding of the evolution of this mutualism, as well as the evolution of termite sociality and disease resistance.
Acknowledgements

I’d like to acknowledge and thank the following individuals who have contributed both directly and indirectly to this body of work.

First and foremost, I’d like to thank my advisor, Dr. Rebeca Rosengaus. Dr. Rosengaus is wonderfully supportive of her graduate students and is always willing to take the time to discuss ideas and help when a problem is particularly perplexing. I have really appreciated all of her investment into me academically and personally.

Dr. Edward Jarroll and Dr. Veronica Godoy-Carter for their excellent input on my research and their generous sharing of research equipment.

Dr. Mark Bulmer for teaching me many of the techniques that were imperative to the research in this paper. Additionally, Dr. Bulmer had insightful and wise comments for my research and school in general.

Marielle Postava-Davignon and Tamara Hartke for helping me to develop research ideas, trouble-shoot problems, and perfect lab techniques. Along with Marielle and Tammy, I’d like to thank Daniel Blustein and Rebecca Westphal, the other members of the graduate student writing club, who have read and critiqued many sections of this thesis.

Ryan Benson for helping me to learn the Bradford assay technique and Alimatu Sulemana for assistance with culture techniques.

Alla Shnayderman for being an incredibly meticulous researcher and for bringing so much enthusiasm to her work. In addition to Alla, I’d like to thank all the other undergraduate students in the Rosengaus Lab, in particular: Brian Lejeune, Casey Hamilton, Patrick Henrick, and Leon Delalio.
Dr. Jim Traniello and Dr. Mark Huyler for their generous sharing of research materials, as well as Dr. John Breznak for his advice on protist cultures.

The administrators of Redwood East Bay Regional Parks District and Huddart Park for allowing us to collect termite colonies on their properties.

The National Science Foundation funded GK-12 program, which has provided me funding for my two years at Northeastern University.

Last, but not least, I’d like to thank Mark Whitaker, Madison Bianca, and my friends and family who have not only supported me, but have been very patient with me as I ramble on about the wonderful world of termites.

This research was funded by NSF CAREER award (DEB 0447316, Rosengaus RB, PI) and an NSF REU supplement 2009 that covered salary for Alla Shnayderman.
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<tr>
<td>βGlu</td>
<td>β-1,3 Glucanase</td>
</tr>
<tr>
<td>βGRP</td>
<td>β-1,3 glucan recognition protein</td>
</tr>
<tr>
<td>cDNA</td>
<td>Cloned deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CM-curdlan RBB</td>
<td>Carboxymethyl Curdlan Remazol Brilliant Blue</td>
</tr>
<tr>
<td>GDL</td>
<td>D-Glucon-1,5 lactone</td>
</tr>
<tr>
<td>GNBP</td>
<td>Gram negative binding protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MWU</td>
<td>Mann-Whitney U</td>
</tr>
<tr>
<td>NaAc</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PDA</td>
<td>Potato dextrose agar</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>Tw80</td>
<td>Tween 80</td>
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<td>WS</td>
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**General Introduction**

Any type of association between members of two different species is loosely termed a symbiosis. These interactions, however, can span a wide range of partnerships, from commensalism to parasitism to mutualism (Kistner, 1982; Hölldobler & Wilson, 1990). When the interactions between the species are beneficial for both partners it is considered a mutualistic symbiosis (reviewed by Moran, 2006). Given the widespread occurrence of microbes throughout our planet (Xu & Gordon, 2003), it is surprising that the impact of microbes on organismal evolution has historically been viewed through pathogenic relationships, while beneficial symbioses have only recently garnered more attention (Ruby et al., 2004; Cash & Hooper, 2005). Most organisms on this planet, regardless of their taxonomic classification, are hosts to an astonishing number of microbial organisms. For example the number of bacterial cells in a human adult far exceeds the number of human cells (Savage, 1977; Berg, 1996) and the number of bacterial genes within a human body may actually exceed the number of human genes (Xu & Gordon, 2003). Thus, any organism can be considered a biome rather than an independent entity. In termites for example, mutualistic gut microorganisms are estimated to comprise between 40-60% of a termite’s mass (Odelson & Breznak, 1983; Bignell, 2000). In many other animals, the vast majority of symbiotic microbes also reside in the intestinal tract (Cash & Hooper, 2005) and these microbes may perform essential roles for their hosts, such as: a) the breakdown of plant polysaccharides (Gibson & Roberfield, 1999; Hooper et al., 2002), b) the synthesis of certain vitamins (Hill, 1997) and c) protection from pathogens (Haine 2007; Lombardo, 2008). The focus of several recent studies, including this one, has been on enhanced pathogen resistance conferred to the
host by its mutualistic microbiota (Currie et al., 1999; Oliver et al., 2002; Scarborough, 2004; Kaltenpoch et al., 2005; Scott et al., 2008).

One way in which symbiotic microorganisms can enhance their host’s resistance against pathogens is by enhancing the host’s production of defensive metabolites (Hooper et al., 2002; Cash & Hooper, 2005; Lombardo, 2008). For instance, studies on gnotobiotic (germ-free) mice have shown how important resident bacteria are to host immunity, as the symbiotic gut microbes of mice stimulate the expression of angiogenin-4, a strongly bactericidal protein produced in the mammalian gut. This protein is an effective killer of gram positive bacteria. In gnotobiotic mice, angiogenin-4 never reaches the high expression levels seen in normal mice, which in turn could influence the development of the innate immune system (Cash & Hooper, 2005). In addition to the production of defensive metabolites, symbiotic microbes are also known to synthesize their own bioactive compounds that are used by the host for defense (Moran, 2006). An illustration of this involves the production of the toxin, pederin, by a bacterial symbiont of the Paederus beetles (Kellner, 2001). Pederin deters predation on the beetle larvae from predators such as the wolf spider (Kellner & Dettner, 1996). Similarly, it has been suggested that a toxin produced by a bacteriophage in the aphid symbiont, Hamiltonella defensa, may be the mechanism by which the symbiont confers protection to its pea aphid host against parasitoid attack (Oliver et al., 2003). These are just a few of the many examples of symbiont-mediated pathogen defense (for more examples see Chapter 1) that illustrate the importance of understanding not only the mechanisms of disease resistance of the host organism, but also of its mutualistic symbionts (Haine, 2007).
The role of symbiont-mediated protection, while important to the individual, has even broader implications in social animals. Mutualistic microbes are hypothesized to have played a key role in the development of group living (Cleveland et al., 1934; Troyer, 1984; Lombardo, 2008). Troyer (1984) proposed that the need to obtain cellulolytic microbes from conspecifics influenced the development of sociality in herbivores. More recently this hypothesis has been expanded to include not only nutritional benefits of symbionts, but protective benefits as well (Lombardo, 2008). Phylogenetically basal termites (i.e. “lower” termites) are an ideal organism with which to test this hypothesis, as they are not only social (Wilson, 1971) but they live in obligate mutualistic symbiosis with a wide array of hindgut bacteria and protozoa (reviewed in Breznak, 2000; Inoue, 2000; Brune, 2006). Termite gut symbionts have been well-studied and have for decades served as a textbook example of mutualism, yet most research done on termite symbionts has focused on the nutritional relationships between termites and their gut microbes (Cleveland, 1925a; Trager, 1932; Yamin & Trager, 1979; Yamin, 1980, 1981; Breznak, 1973, 1985). To date, very little research has tackled the role that these gut microorganisms may have on host disease resistance.

The dampwood termite, Zootermopsis angusticollis, provides a unique test organism for exploring symbiont-mediated pathogen defense within the context of eusociality. Not only are these termites relatively large and easy to work with, they have been used as model organisms in a multitude of disease resistance and transmission studies (Rosengaus et al., 1998a, 1998b, 1999a, 2000, 2001, 2003, 2004, 2007; Traniello et al., 2002). Additionally, Z. angusticollis like other lower termites live in one-piece nests which function as both shelter and food (reviewed in Noirot & Darlington, 2000).
Due to these living habits, they face intense pathogenic pressures (Blackwell & Rossi, 1986; Ljutikova, 1990; Rosengaus & Traniello, 1997; Rosengaus et al., 2003). Living in microbe-rich environments predisposes termites to fungal infections (Blackwell & Rossi, 1986; Rosengaus & Traniello, 1997; Rosengaus et al., 2003). Additionally, termites engage in frequent close-range mutual interactions that can foster both disease transmission and symbiont exchange amongst members of the colony (Freeland, 1979; Troyer, 1984). Although the role of disease in the evolution of sociality has traditionally emphasized costs of group living (Freeland, 1979; Brown & Brown, 1986; Nunn et al., 2000), there has been limited consideration of the advantages of controlling infection that accompany colonial life (Rosengaus et al., in press). Recent research however is indicating that social interactions may indeed prevent or help control infection (Rosengaus et al., 1998a; Schmid-Hempel, 1998; Wilson & Reeson, 1998; Rosengaus & Traniello, 2001; Traniello et al., 2002; Cotter & Wilson, 2004; Ugelvig & Cremer, 2007; Cremer et al., 2007; Fefferman & Traniello, 2008). In the case of termites, mutual grooming, coprophagy, proctodeal feedings (the ingestion of fecal droplets), and/or mouth-to-mouth regurgitation (oral trophallaxis) could intensify pathogen-related mortality, as suggested by Rosengaus, et al. (1998a, 2000). Alternatively, these same behaviors could reduce disease susceptibility by promoting the social transfer of immune factors, elicitors or proteins that would render the recipient less susceptible to disease (Traniello et al., 2002; Rosengaus et al., in press). Lower termites transfer symbionts during proctodeal feedings (Wilson, 1971) and if these symbionts provide not only nutritional support, but also pathogen resistance, then an even stronger case can be made for the influence of mutualistic symbionts on the evolution of sociality: sharing
symbionts could have become highly adaptive if nestmates benefited from improved abilities to breakdown cellulose while increasing their resistance against pathogenic microorganisms.

In this thesis, symbiont-mediated pathogen defense is explored through both in vivo and in vitro experiments using the termite, *Z. angusticollis*, and the entomopathogenic fungi *Metarhizium anisopliae*. In Chapter one, in vivo studies examine the role of symbionts in termite susceptibility to fungal pathogens by comparing the survival of termites with and without their hindgut microbial consortia. In Chapter two, an enzyme of symbiont-origin is identified. This enzyme, β-1,3 glucanase, has known pathogen defense functions in other organisms (Leubner-Metzger & Meins, 1999; Bulmer et al., 2009) and may provide pathogen defense in *Z. angusticollis* as well. Lastly in Chapter three, a series of in vitro experiments confirm the strongly fungistatic nature of the termite gut and the role of symbiont-produced β-1,3 glucanases in gut fungistasis is further explored.
Chapter 1

Symbiont-mediated protection against fungal infection in the dampwood termite, Zootermopsis angusticollis: In vivo experiments.
**Introduction**

Recently, there has been a surge of interest in microbial symbionts across diverse taxonomic groups. Microbes make up the largest (and least understood) sector of species diversity on our planet (Stahl and Tiedje, 2002). Many of these microbes perform a variety of functions for an array of host organisms, including humans (Stahl and Tiedje, 2002). The roles of these symbiotic microbes include (but are not limited to): the synthesis of necessary vitamins; the breakdown of indigestible plant polysaccharides; induction of normal tissue development and protection from pathogens (Hill, 1997; Gibson and Roberfroid, 1999; Hooper et al., 2002; Haine 2007). Insects, in particular, are good examples of where the effects of symbiont-mediated pathogen defenses are evident (see *symbiont-mediated pathogen defense* section below).

The present research examines, in a novel way, the mutualistic symbiotic interactions between the dampwood termite, *Zootermopsis angusticollis* and its hindgut microbiota. Historically, this microbial community has been considered essential for the host’s nutrition (Cleveland, 1924; 1925a). Yet, the present work differs from previous research in that it focuses on a pathogen defense role for symbionts. Termites are ideal study organisms for this research, as they live in microbe dense environments predisposing them to fungal infection (Blackwell & Rossi, 1986; Rosengaus & Traniello, 1997; Rosengaus et al., 2003) and have a well-studied obligate mutualism with their gut symbionts (as reviewed in Inoue 2000; Bignell, 2000; Brune, 2006). Additionally, previous research has shown that the symbiont-filled gut is extremely inhospitable to fungal conidia (Kramm & West, 1982; Yanagawa & Shimizu 2007), and the suggestion
has been made that unknown factors of symbiont origin inactivate the conidia (Boucias et al., 1996; Siderhurst et al., 2005, Chouvenc et al., 2009).

**Structure of the wood-feeding termite gut and associated microorganisms**

The gut of the lower termite is divided into three sections: a short foregut, a midgut, and a hindgut (fig. 1; Noirot, 1969). Each compartment provides microhabitats with unique biochemical parameters (Brune, 2006). A diverse assemblage of gut microorganisms lives throughout the gut lumen and on the epithelial surfaces (Bignell, 1984). The foregut is a simple tube covered with a thin layer of cuticle, which leads into the midgut (Noirot & Noirot-Timothée, 1969). The midgut is not protected by cuticle, instead it is lined by a peritrophic membrane (Terra, 1990; Brune, 2006) and it is the primary site of enzyme secretion (Noirot & Noirot-Timothée, 1969). The lumen of this section houses prokaryotic microorganisms including Actinomycetes bacteria in many species, which are of unknown function (Bignell, 2000). The pH of the foregut and midgut ranges from 6.8 near the esophagus to 5.2 in the midgut (Noirot & Noirot-Timothée, 1969).

The last and largest section of a termite’s digestive tract, referred to as the hindgut, houses the protists in lower termites and it constitutes the majority of the termite’s weight (Bignell, 2000). In addition to the protists inhabiting the hindgut, the cuticle of the hindgut epithelium is intimately associated with a variety of bacteria (Brune, 2006). Within the hindgut the anaerobic protists together with prokaryotic microorganisms are localized within a dilated region called the paunch (Noirot & Noirot-Timothée, 1969; Fig 1). The hindgut, like the esophagus, has a relatively neutral pH of
6.8 (Noirot & Noirot-Timothée, 1969) and while originally thought to be completely anoxic, in reality only the central portion of the paunch is anoxic with the outer epithelium being oxic (Ebert & Brune, 1997). The protists that reside there belong to three orders: Oxymonadida, Trichomonadida, and Hypermastigida (fig. 2; Inoue et al., 2000) and these protozoa provide a microhabitat to distinct bacteria that do not occur elsewhere in the termite gut (Brune, 2006). Indeed, all hindgut protists that have been investigated have intimate associations with prokaryotes living on or within the protist cell itself (Brune, 2006).

Classic experiments done by Cleveland in the 1920’s established the importance of the obligate anaerobic hindgut microbiota to termite survival (Cleveland, 1924, 1925a, 1925b, 1928). A major breakthrough in the understanding of some of these protists’ roles came with Yamin’s axenic isolation of the Z. angusticollis protists, Trichomitopsis termopsidis (fig. 2; Yamin, 1978) and Trichonympha sphaerica (fig. 2; Yamin, 1981). He demonstrated that cellulose digestion by protists was independent of their associated bacteria, hence clearly establishing the role of protists as cellulolytic symbionts. Since then, the association of flagellated protists and termites has become a textbook example of obligate mutualistic symbiosis (Inoue et al, 2000). However, this simplistic view has been under revision and scrutiny with the advent of molecular techniques revealing endogenous termite cellulases (Watanabe et al., 1998). A more realistic picture is one in which the protozoan community contributes to the termite’s own cellulase activity, which is insufficient to keep the termite well nourished and alive (Slaytor, 2000). Despite intense research, there is still relatively little known about the full depth of the mutualism
between lower termites and their protists (Inoue et al., 2000) and possibilities beyond a nutritional symbiosis have yet to be explored.

**Termite Pathogen Response**

Pathogens have played an important role in the evolutionary ecology of social insects, including termites (Rosengaus et al., 1998a, 2000, 2003; Cremer et al., 2007). Termites live in dense colonies with frequent social interactions, which increase the potential for rapid disease transmission (Rosengaus et al., 1998a, 2000; Rosengaus and Traniello, 2001; Fefferman et al., 2007). In order for sociality to have evolved, the benefits of group living must have outweighed the costs (Alexander, 1974). Indeed, termites have mitigated the costs of pathogen transmission through a number of adaptations including immunological, behavioral, and biochemical responses (reviewed below). Similar mechanisms have also been shown in the social Hymenoptera (reviewed in Schmid-Hempel, 1998).

**a) Immunological responses**

The defenses of the insect innate immune system include humoral and cellular components and the ability to discriminate between self and invading non-self (Schmid-Hempel, 1998). As part of the humoral response, insects use pathogen recognition proteins to recognize and bind to invading pathogens (reviewed in Schmid-Hempel, 1998; Janeway & Medzhitov, 2002); this recognition can initiate immune responses in the hemolymph (Gobert et al., 2003; Ferrandon et al., 2004). The cellular response aspect of the immune system includes direct phagocytosis of small particles (i.e. bacteria)
and the encapsulation of larger parasites such as nematodes, parasitoid eggs, etc. (reviewed in Schmid-Hempel, 1998; Loker et al., 2004). Although insects, in general, are not thought to have an adaptive immune response (Schmid-Hempel, 1998; Janeway & Medzhitov, 2002), there is evidence that after an “immunizing” exposure to a sub-lethal dose of bacteria or a fungal pathogen, termites were better able to survive a second exposure to that same pathogen (Rosengaus et al., 1999a, 2007; Traniello et al., 2002).

b) Behavioral responses

Before pathogens can even invade the insect body, they must circumvent the external biochemical and behavioral responses. For example, termites that are in direct contact with lethal dosages of fungal conidia engage in a vibratory alarm behavior. This vibratory alarm causes nestmates not in contact with conidia to move away from the source of infection (Rosengaus et al., 1999b). Grooming, another behavior in response to pathogens, is one of the most effective termite mechanism against fungal infection. Furthermore, allogrooming (nestmate grooming nestmate) is a more successful mechanism for removing fungal conidia than is self-grooming (Rosengaus et al., 1998a, 2000: Yanagawa & Shimizu, 2007). Yet, the groomer risks becoming infected as it ingests conidia removed from the cuticle of infected nestmates. These conidia could potentially invade the groomer’s alimentary tract, most of which is lined with cuticle, the main substrate that triggers conidia germination in some entomopathogenic fungi (Dillon & Charnley, 1991; Charnley & Leger, 1991). Despite the apparent costs to the groomer, previous termite research indicates that ingested conidia become inactivated after their
passage through the insect gut (Kramm & West, 1982; Yanagawa & Shimizu, 2007; Chouvenc, 2009).

c) Biochemical responses

In addition to immunological and behavioral adaptations to resist pathogens, termites have evolved several biochemical mechanisms as well (Batra & Batra, 1966; Rosengaus et al., 1998b, 2000, 2004). Sternal gland secretions from Z. angusticollis significantly inhibit the germination of M. anisopliae conidia (Rosengaus et al., 2004). While sternal gland secretions are known for their role in termite trail following (Grassé, 1986), Rosengaus et al. (2004) suggested that these compounds might have evolved secondarily to function in an antimicrobial role. In addition to bodily secretions, the feces of Z. angusticollis has fungistatic properties and is used to line the nest possibly as a means to reduce fungal growth within the nest (Rosengaus et al., 1998b). The liquid feces of lower termites contains abundant protists (Andrew, 1930), and β-1,3 glucanases of symbiotic origin (see Chapter 2). There is potential that the fungistatic properties generally observed in lower termite guts (Yanagawa & Shimizu, 2007; Chouvenc et al., 2009) and observed in Z. angusticollis feces (Rosengaus et al., 1998b) are due to the presence of hindgut symbionts themselves or their metabolites.

Symbiont-mediated pathogen defense

In the study of symbiotic relationships, the effect of a third party parasite is often ignored, despite the important selection pressures that parasites may place on both members of an established mutualistic symbiosis (Little & Currie, 2009). The fungus-
growing ants (Attini: Formicidae) provide a perfect illustration. Fungus cultivating ants live in obligate mutualism with fungi of the family Lepiotaceae (Chapela et al., 1994; Currie et al., 1999; Currie, 2000). The fungal gardens of these ants are at risk of invasion by virulent parasitic fungi of the genus *Escovopsis* (Currie et al., 1999). However, these ants are also host to epibiotic Actinomycete bacteria, which are located in specific regions of the ant’s body depending on the ant genus. These Actinomycetes produce antifungals that specifically inhibit the growth of the parasitic fungi, but not the mutualist fungi of ants (Currie et al., 1999). Another example includes the southern pine beetle, *Dendroctonus frontalis*, which lives in a similar symbiosis with the fungus, *Entomocorticium sp*. Like the fungus-growing ants, the southern pine beetle has special structures on its body to house antibiotic producing Actinomycetes, which help to maintain fungal gardens free of pathogenic invaders (Scott et al., 2008).

Symbioses for invertebrates go beyond the protection of food resources from potential competitors, as some invertebrates use epibiotic mutualists to protect themselves or their embryos from fungal pathogens. The European Beewolf wasp, *Philanthus triangulum*, have *Streptomyces* bacteria in specialized glands on their antennae. They liberally apply these bacteria to brood cells prior to oviposition, which help protect the brood from fungal infection (Kaltenpoch et al., 2005). Another example of embryo protection by symbionts can be found in the shrimp, *Palaemon macrodactylus*. The embryos of these shrimp are resistant to fungal infection when they themselves are coated with a commensal *Alteromonas* sp. bacterial strain. The *Alteromonas* sp. produces a 2,3-indolinedione compound that inhibits the pathogenic fungus. Removal of this bacterial symbiont results in the quick death of the shrimp embryo (Gil-Turnes, 1989).
Symbionts not only function as external defenders (Gil-Turnes, 1989; Currie et al., 1999; Scott et al., 2008) but they can also enhance the robustness of the host’s own immune system by adding yet another layer of defense against pathogens (Kitano & Oda, 2006). For example, a facultative bacterial endosymbiont, *Regiella insecticola*, of the pea aphid, *Acyrthosiphon pisum*, augments host resistance to a fungal pathogen; not only increasing the direct fitness of the pea aphid, but also its indirect fitness by reducing sporulating aphid cadavers in proximity to its genetically identical neighbors (Scarborough et al., 2004). Pea aphids also receive protection against parasitoid wasps due to the presence of specific facultative bacterial symbionts. Presence of facultative symbionts caused increased mortality of parasitoid larvae, thus increasing survivorship of the pea aphid hosts (Oliver et al., 2002).

While it is known that many gut symbionts provide nutritional support in a variety of organisms (reviewed in Lombardo, 2008), some gut symbionts could also provide protection against pathogens (Savage, 1977; Tannock, 1995; Kitano & Oda, 2006). A case in point is the desert locust, whose normal gut fauna reduce the pathogenicity of *M. anisopliae* (Dillon & Charnley, 1991). In termites, the role of gut microbiota in prevention of foreign bacterial colonization has been examined (Veivers et al., 1982). In the termites, *Nasutitermes exitiosus* and *Coptotermes lacteus*, the removal of the natural gut bacteria with tetracycline allowed for the invasion of the opportunistic pathogen, *Serratia marcescens*. Removal of anaerobic symbionts with hyperbaric oxygen, instead of antibiotics, caused an increase in the resident aerobe-tolerant bacterial fauna, and invading *S. marcescens* was excluded from the termite gut in 6–10 days. The evidence from this study seems to suggest the resident bacteria “out-competed” the invading
bacteria; this is commonly referred to as the ‘competitive-exclusion hypothesis’ (Savage, 1977; Tannock, 1995). However, a competitive-exclusion scenario does not explain the strong fungistatic activity observed in the hindguts of lower termites (Yanagawa and Shimizu, 2007; Chouvenc, 2009). To test for the in vivo benefits of termite symbionts against the fungal pathogen, M. anisopliae, termites were defaunated with hyperbaric oxygen, and exposed to a fungal conidia suspension or a control solution. If hindgut symbionts provide a fungistatic benefit to their host, then defaunated termites will be significantly more susceptible to mycosis then controls. This is the first study to focus on the intersection of symbionts and fungal resistance.
Materials and Methods

Termite collection

Colonies of the dampwood termite, *Zootermopsis angusticollis*, were collected from the Huddart Park (San Mateo County) and the Redwood East Bay Regional Park in Oakland, CA in July of 2003 and more recently in August 2008. Lab-reared colonies were established by pairing male and female alates selected from field-collected colonies. All colonies have been maintained in our USDA approved containment room at 25°C inside closed plastic containers and are given water and birch wood as food *ad libitum*.

Defaunation procedure

The majority of hindgut termite symbionts are anaerobic (Breznak, 1983). Thus, through the use of an oxygenation treatment, researchers have previously eliminated all of the flagellated protozoan community while leaving a portion of the bacterial community intact without injuring the termite (Cleveland, 1925b; Messer & Lee, 1989). In order to defaunate termites, the oxygenation system depicted in fig. 3 was devised. This procedure was adapted from Cleveland (1925b). Nymphs and psuedergates of *Z. angusticollis* were randomly selected from field collected or lab-reared colonies and then placed inside open-ended 15 ml plastic culture tubes. Tubes were lined with a strip of paper towel moistened with 300 μl of sterile water. The tubes were then plugged with moistened cotton-balls and placed into a steel pressure canister (Sure-Shot Atomizer©). The canister containing the termites selected for defaunation was subsequently connected to an oxygen tank, which was first flushed with oxygen for 60 sec. to remove residual
The steel canister was then sealed as oxygen from the tank continued to flow into it, until a pressure of 40 psi was reached. The termites (n=10/tube) remained under high-pressure oxygen for one hour and then the canister was depressurized (but not opened). The termites remained under these concentrated oxygen levels (≈97%) for 24 hours, post depressurization. Control faunated termites were subjected to identical manipulations as those of defaunated nestmates with the exception that they were not exposed to oxygen. They were, however, subjected to 40 psi of pressurized air (pressurized by using a bicycle pump) and also remained in the pressurized steel canister for one hour. The canister was then depressurized and the termites remained in the closed container for 24 hours. After such treatments, one to two termites from each group were randomly selected for confirmation of the effectiveness of the oxygenation treatment in defaunation, relative to termites in the control treatment (fig. 4). Confirmation was achieved by examining 10 µl of the hindgut fluid of a dissected termite on a hemocytometer using a compound light microscope (Nikon Eclipse E400) at 400X magnification.

*Exposure to the fungal pathogen M. anisopliae*

After undergoing defaunation or the pressure control treatments, termites were allowed to walk freely for one hour inside a Petri dish (60 x 15 mm) lined with filter paper (Whatman #5) moistened with 374 µl of either a 1x10^5 conidia/ml suspension of the entomopathogenic fungus *Metarhizium anisopliae* suspended in a 0.1% Tween 80 (Tw80) or a 0.1% Tw80 solution lacking conidia (Rosengaus et al., 1998a). In addition to the control and defaunate treatments, a group of naïve termites (taken directly from the nest) were also exposed to a Tw80 or a 1x10^5 conidia/ml suspension. These naïve groups
were established to determine the effects that the additional manipulations resulting from the oxygenation and pressure control treatments may have beyond that of infection alone. Termites were maintained in groups of ten during exposure to conidia or Tw80. Following exposure, they were transferred in these same groups to sterile Petri dishes (60 x 15 mm) lined with clean filter paper (Whatman #1) and given water ad libitum and maintained at 25°C. Termite survival was monitored daily for 21 days post-exposure. Dead termites were removed, surface sterilized with hypochlorite (6.0%), and plated on potato dextrose agar (PDA) plates to confirm if the cause of death was due to M. anisopliae infection (Rosengaus & Traniello, 1997).

**Fat Quantification Assay**

Previous research has demonstrated the important role of protists on termite nutritional status (Cleveland, 1925a) and the fat body of insects has been linked to production of immune-related compounds (reviewed in Hoffman, 1995). Thus, the fat quantification assay helped to infer whether starvation played a role in the survival of defaunated individuals following exposure to fungal conidia. Termites, in groups of 10, first underwent defaunation or pressure control treatments as previously described. Termites were housed similarly in plastic Petri dishes (60 x 15 mm) lined with filter paper (Whatman #1) and given water ad libitum. On the 6th day post-treatment, termites were sacrificed to compare their fat content, as previous *in vivo* experiments indicated that defaunated and conidia exposed termites reached 50% mortality on this day. Defaunated and pressure control (faunated) termites were cold immobilized and decapitated. The decapitated termites were allowed to dry under a fume hood for 3-4
days. Once termite cadavers were completely dried-out, they were individually weighed and then placed in glass vials containing 95% diethyl ether (Fluka BioChemika). The vials were sealed and then shaken at 100 RPM for 24 hours to allow the diethyl ether to dissolve the fat. The diethyl ether was then removed and the termite cadavers were allowed to air dry for another 24 hours before being individually reweighed (Ellers, 1996; Doums et al., 2002). In this way the mass difference of dried termites before and after the dissolution of their fat tissue was calculated.

Statistics

To determine the effect of defaunation on disease susceptibility, survival parameters were compared across the following treatments using survival analyses (SPSS, v. 17.0): naïve individuals exposed to Tw80, naïve individuals exposed to a 1x10^5 conidia solution, faunate pressure control individuals exposed to Tw80, faunate pressure control individuals exposed to a 1x10^5 conidia solution, defaunate individuals exposed to Tw80, and defaunate individuals exposed to a 1x10^5 conidia solution. The measured parameters included: survival distributions, percent survival at the end of the 21 day census, the median survival time (LT₅₀, the length of time it takes for 50% of the individuals undergoing a treatment to die; Kaplan-Meir Test; SPSS 17.0) and the relative hazard ratio of death (Cox Proportional Hazard Regression Analysis; SPSS 17.0). A Mann-Whitney U (MWU) test was used to quantify differences in fat content between defaunated and faunated termites (SPSS 17.0). The Bonferroni correction was applied for multiple pairwise comparisons setting a more conservative significance level of p≤ 0.05/n, where n=the number of pairwise comparisons.
Results

The Cox Proportional Regression Model showed that colony of origin (n=3) was not a significant and independent predictor of termite survivorship (Wald statistic (WS) =4.9, df=2, p >0.05, fig. 5). Therefore, data from all colonies were pooled for further statistical analyses. Termite exposure (fungal conidia or Tw80) and treatment (naïve, defaunated, or faunated) were significant and independent predictors of termite survival (WS=168.4, df=2, p<0.001; fig. 6; WS=194.7, df=1, p<0.001; fig. 7; respectively; Cox proportional hazard regression; SPSS 17.0). The combination of treatment and exposure was also a significant predictor of termite survivorship (WS=284.7, df=5, p<0.001, Cox proportional hazard regression; SPSS 17.0). The effect of each of these variables is discussed, in turn, below.

Effect of exposure to M. anisopliae on termite survivorship

After controlling for the effect of colony of origin and treatment, exposure to fungal conidia was a significant and independent predictor of termite survivorship (WS=194.7, df=1, p<0.001, fig. 6; Cox proportional hazard regression; SPSS 17.0). Relative to Tw80 controls (n=423), conidia exposed termites (n=416) had 4.8 times the hazard ratio of death (Cox proportional hazard regression; SPSS 17.0).

Effect of treatment on termite survivorship

Treatment was a significant and independent predictor of termite survivorship (WS=168.4, df=2, p<0.0001; fig 7). Faunated pressure control individuals (n=360) experienced a 2.6 times higher hazard ratio of death (95% CI 1.9-3.7) relative to the naïve
individuals (n=117), even after controlling for the effects of colony of origin and exposure to conidia. Defaunated individuals (n=362) had an increase of 6.1 in the hazard ratio of death (95% CI 4.3-8.5) relative to naïve termites (fig. 7; Cox proportional hazard regression; SPSS 17.0). These results show that the defaunation protocol itself caused mortality during the 21 day census, relative to naïve nestmates. However, we were interested in testing the effect that the presence or absence of hindgut microbiota had on fungal disease susceptibility. Therefore, the appropriate control group for comparison is the pressure treated faunated individuals as their group underwent similar manipulations as the defaunated individuals.

More detailed analyses were performed after excluding the naïve treatment, in order to better control for the effects of added handling and pressurization. After exclusion of naïve individuals, treatment while controlling for the effects of exposure and colony of origin remained an independent and significant predictor of termite survivorship (WS=121.0, df=1, p<0.0001), indicating that oxygen had an effect beyond the stressors of manipulation and handling. All further analyses were conducted with the exclusion of the naïve treated individuals. The hazard ratio of death for defaunated termites relative to faunated pressure treated termites, while controlling for the effects of exposure and colony of origin was 3.0 (95% CI 2.4-3.6; Cox proportional hazard regression; SPSS 17.0).

*Effect of treatment on disease susceptibility*

Pairwise comparisons of the combined effect of treatment and exposure revealed significant differences between all groups (faunate Tw80, faunate 10^5, defaunate Tw80,
Defaunated Tw80 individuals (see table for all n values) experienced significantly higher mortality than faunated Tw80 individuals (Log Rank $X^2=107.1$, $p<0.0001$, fig. 4, Kaplan-Meier test; SPSS 17.0). The combination of defaunation and fungal exposure significantly increased termite mortality relative to fungal exposure with pressure treatment alone (Log Rank $X^2=62.2$, $p<0.0001$; fig. 8, Kaplan-Meier test; SPSS 17.0). Defaunated fungal-exposed termites were 2.2 times as likely to die when exposed to conidia, relative to faunated fungal-exposed termites (95% CI 1.74-2.75; Cox proportional hazard regression; SPSS 17.0). This illustrates the importance of termite hindgut symbionts to fungal resistance. Not only did the defaunated fungal conidia-exposed termites experience significantly higher mortality compared to all other groups, but by day 15 of the census they had experienced 100% mortality, whereas 20% of the faunated fungal-exposed individuals were still alive by the end of the 21 day census (fig. 8 & table 1).

The defaunated Tw80 termites had 6.6 times the hazard ratio of death relative to faunated Tw80. The defaunated fungal-exposed termites had the highest hazard ratio of death (20.7) relative to the faunated Tw80 individuals. Fungal-exposed faunated termites had a hazard ratio of death 9.4 times that of their Tw80 counterparts (table 1).

**Post-mortem confirmation analysis**

In order to confirm cause of death, termites were surface sterilized and plated on PDA. Defaunated conidia-exposed termites had similar *M. anisopliae* confirmation rates as compared to faunated conidia-exposed (table 1). None of the faunated or defaunated Tw80-exposed termites confirmed for *M. anisopliae*. Many of the conidia exposed
termites succumbed to other fungi, including *Aspergillus* sp. It is possible that
*Aspergillus* is able to out compete *M. anisopliae* in dually infected termites, as is this case
in leaf-cutter ants (Hughes and Boomsma, 2004).

*Fat quantification assay*

The removal of hindgut symbionts via oxygen in *Z. angusticollis* did not appear to
cause malnutrition, as measured by fat content, six days post-defaunation treatment. The
two colonies tested showed a significant difference in fat content (MWU=145.5, p<0.05; 
fig. 9; SPSS 17.0). This is not unusual as individuals from different colonies often vary
greatly in size (personal observation). However, there was not a statistically significant
difference in fat quantity between defaunated and faunated control termites in either
colony (fig. 9). In colony 8, the median fat quantity for faunated termites was 7.25 mg
while the median fat quantity for defaunated termites was 6.30 mg (MWU; SPSS 17.0). 
In colony 9, the median fat quantity for faunated termites was 1.15 mg while the median
fat quantity for defaunated termites 0.75 mg (MWU; SPSS 17.0). Although defaunated
termites tended to have a lower mass than their faunated counterparts six days post
oxygenation, this difference was not statistically significant. Therefore, it is unlikely that
differences in fat quantity played a large role in the differential susceptibility to fungal
infection between defaunated termites and faunated termites.
Discussion

These data demonstrate, for the first time, that faunated termites are significantly less susceptible to mycosis than their defaunated nestmates (fig. 8). As lower termites depend on their flagellated protozoa for much of their nutrition (Cleveland, 1924, 1925a; Eutick et al., 1978), this study focused on the survival analysis towards the earlier days following fungal exposure. Therefore the effect of malnutrition and starvation, which takes place at approximately three to four weeks after termites are deprived of all cellulose material (Cleveland, 1925a), was minimized. As lower termites have endogenous cellulases and are not entirely dependent on their gut microbes for nutrition (Watanabe et al, 1998; Slaytor, 2000), they should obtain a small amount of nutrition from their filter paper substrate. Our fat quantification assay indicated that after 6 days post-exposure (the median survival time for defaunated conidia-exposed termites), there was not a significant difference in the fat content between the two treatments. As many insects depend on their fat body for production of immune compounds (as reviewed in Hoffman, 1995), it was important that differences in fat content of our experimental animals were minimized.

Upon defaunation, many of the termites in the Tw80 treatment succumbed to Serratia sp., Aspergillus sp., and Psuedomonas sp. These pathogens are typically opportunistic (Bucher & Stephens, 1957; Boucias & Pendland, 1998). The susceptibility of oxygen-defaunated termites to Serratia sp. and Psuedomonas sp. in this study is in contrast to the results of Vievers et al. (1982). In that study, oxygen defaunation combined with exposure to Serratia via ingestion did not increase mortality over oxygen defaunation alone. However, they did find in the lower termite, Coptotermes lacteus, that
*Serratia* transiently invaded the hindgut of oxygen defaunated individuals, but were undetectable within 6-10 days. It should be pointed out that Vievers’ et al. (1982) study focused on the incidence of *Serratia* in the gut (i.e. the culturability of *Serratia* from the hindguts of sacrificed termites) and did not examine the cause of death of the deceased termites. The inability of foreign bacteria to take hold in oxygen defaunated termites may be due to the increase in aerobe tolerant bacteria after oxygen treatment (Veivers et al., 1982). This would be consistent with the protective effect that native gut bacteria have against invading bacteria by out-competing and/or excluding them (Freter, 1956). However, our data suggest that other factors are at work. In our study, the abundant aerobe tolerant bacteria remaining in the gut were not able to preclude opportunistic pathogens. Additionally the loss of the strictly anaerobic gut microbes coincided with a decreased ability to resist *M. anisopliae*. It seems probable that the protists and strict anaerobes removed in this experiment are important in pathogen defense.

Protective microbe interactions with termites could have arisen as a consequence of competition between native well-established microbiota and invading microbes. The “interests” of the mutualistic microorganisms are likely opposed to the interests of the invading pathogens, since they are competing for resources within the host. As the fitness of the obligate mutualist is directly linked to the survivorship of its host, there is a high probability of conflict between the resident symbiont and invading pathogen. The invading pathogen may reduce host fitness by reducing fecundity or killing the host, consequently reducing symbiont fitness as well (Rigauld & Haine, 2005). In addition to the conflict over host fitness, there is the potential conflict over resources within the host. This conflict could potentially be resolved if the symbiont community provided
protection to its host against the pathogen (Haine, 2007). This is the case in the desert locust, *Schistocerca gregaria*, whose gut bacteria produce toxic phenols that are both antibacterial and antifungal (Dillon & Charnley, 1995).

Several lines of evidence, including results from Chapter 3 of this thesis, indicate that termite guts may also have antimicrobial properties. Conidia of *M. anisopliae* in the alimentary tracts of termites do not germinate (Siderhurst et al., 2005; Yanagawa & Shimizu, 2007; Chouvenc et al., 2009) even when allowed prolonged contact with the cuticle lining the gut (Chouvenc et al., 2009). The hostile environment of the alimentary tract is not likely to be due to environmental factors such as pH or oxygen levels. The pH of the paunch is within the range of *M. anisopliae* germination (Brune et al., 1995; Dillon & Charnley, 1986) and while an oxygen deficit is normally inhibitory to the germination of conidia (Cochrane et al., 1963), the termite gut is not completely anoxic as the perimeter regions are oxic (Ebert & Brune, 1997). Not only is the gut of living termites fungistatic, but this effect also persists even after termite death. Examinations of termite cadavers two days post-mortem showed that hyphal growth of *M. anisopliae* was generalized over the body with the exception of the hindgut lumen. This suggests that chemical activities likely to be of termite or mutualist origin exist in the gut, which remain effective after termite death (Chouvenc et al., 2009).

Despite these lines of evidence indicating that gut symbionts of lower termites may play a role in host defense, this is the first empirical test focusing on the *in vivo* effects of symbiont removal and fungal susceptibility of the host. This study points to a pathogen-defense role for termite gut symbionts. Although the exact nature of this role requires more investigation, the production of β-1,3 glucanases by the same termite
hindgut symbionts (see Chapter 2) removed with oxygen in this study, provides a potential mechanism by which gut microbes inhibit pathogens and enhance host survival
Figure 2: Representatives of the most dominant hindgut protozoa from *Z. angusticollis*.  
A) *Trichomitopsis termopsidis*; Photo Credit*: Zettler, L. A., Olendzenski, L., & Patterson, D.  
B) *Trichonympha sp.*; Photo Credit: Schultheis, K. F.  
C) *Hexamastix sp.*; Photo Credit*: Zettler, L. A., Olendzenski, L., & Patterson, D.  
D) *Streblomastix strix*: Photo Credit*: Brugerolle, G.  

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Enlarged view of open-ended culture tube lined with a moist paper towel and plugged with cotton balls.

11cm x 1.5cm

**Figure 3:** Defaunation set up. Open-ended 10 ml culture tubes plugged with cotton were placed into the pressure vessel (Sure-Shot Atomizer®) and then either pressurized with oxygen using an oxygen tank (defaunated treatment), or pressurized with air using a bicycle pump (faunated pressure control).
Figure 4: Comparison of protists/ml of termite gut fluid for defaunated termites (n=12), faunated control termites (n=12) and naïve termites (n=7). Termite gut contents from 9 colonies were examined for the presence/absence of protozoa post-treatment. There were no significant differences between colonies (Kruskal-Wallis Test $X^2=11.7$, df=8, p=0.167). The protist/ml for all defaunated termites was zero. The remaining boxplots show the median value and interquartile range. The outliers, identified by an open circle, included cases with values between 1.5 and 3 box lengths from the lower edge of the box. Bars with the same letter are not significantly different in pairwise comparisons (by MWU test adjusted with Bonferroni correction ($P > 0.017$), SPSS 17.0).
Figure 5: Effect of colony of origin (A, B, and C) on termite survivorship, while controlling for the effect of exposure (Tw80 and $10^5$ conidia/ml) and treatment (naïve, faunated pressure control, and defaunated). The survival distribution of Colony C and Colony B overlap. Colony of origin was not a significant predictor of survivorship (WS=4.88, df=2, p >0.05, Cox proportional hazard regression; SPSS 17.0).
Figure 6: Effect of exposure (Tw80 and $10^5$ conidia/ml) on termite survivorship, while controlling for the effect of colony of origin (A, B, and C) and treatment (naïve, faunated pressure control, and defaunated). Fungal exposure was a significant and independent predictor of survivorship ($WS=194.7$, df=1, $p<0.001$, Cox proportional hazard regression; SPSS 17.0).
Figure 7: Effect of treatment (naïve, faunated pressure control, and defaunated) on termite survivorship, while controlling for the effect of colony of origin (A, B, and C) and exposure (Tw80 and $10^5$ conidia/ml). Termite treatment was a significant and independent predictor of survivorship ($WS = 168.4$, $df=2$, $p<0.0001$, Cox proportional hazard regression; SPSS 17.0).
Figure 8: Survival distributions of defaunated and faunated termites exposed to either a Tw80 solution or a $1.0\times10^5$ conidia suspension. Different letters indicate significant differences in the time course of survival (p<0.0001)
Table 1: Survival parameters of faunated control and defaunated individuals after exposure to Tw80 or *M. anisopliae* conidia (1 x 10^5). Hazard ratios of death are in reference to faunate control Tw80 treatment, which exhibited the highest survival (• Indicates that LT_{50} was not reached at the end of the 21 day census period). Percent of termites confirming *Metarhizium anisopliae* (% M.A. confirm) as cause of death are shown in the far right column.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>LT_{50} (in days)</th>
<th>% Survival</th>
<th>Hazard Ratio</th>
<th>% M.A. confirm.</th>
</tr>
</thead>
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<td>•</td>
<td>84.5</td>
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<td>Faunate 10^5</td>
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<td>8</td>
<td>19.6</td>
<td>9.4</td>
<td>29%</td>
</tr>
<tr>
<td>Defaunate Tw80</td>
<td>182</td>
<td>14</td>
<td>28.6</td>
<td>6.6</td>
<td>0%</td>
</tr>
<tr>
<td>Defaunate 10^5</td>
<td>180</td>
<td>6</td>
<td>1.1</td>
<td>20.7</td>
<td>35%</td>
</tr>
</tbody>
</table>
Figure 9: Comparison of termite fat quantity (in mg) six days post pressure treatment (faunated control) or pressure treatment plus oxygen (defaunated) in two colonies. Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box. There were no significant differences between the two treatments in either colony 8 or colony 9 (MWU=236.50, p=0.095; U=78.0, p=0.061; SPSS 17.0).
Chapter 2

β-1,3 glucanases produced by the hindgut symbionts of *Zootermopsis angusticollis*
and their potential role in termite pathogen defense
Introduction

β-1,3 glucans are major structural components of fungi (Pitson et al., 1993), plants and some bacteria (Brown & Gordon, 2005). β-1,3 glucans are also common pathogen associated molecular patterns (PAMPs). PAMPs are highly conserved molecular structures located on the cell surfaces of pathogens and are thought to be essential to the survival of the pathogen, yet are not produced by the metazoan host. Some well-studied PAMPs of bacteria include lipopolysaccharides (LPS), peptidoglycans and lipoteichoic acids. Both vertebrates and invertebrates have developed different mechanisms to recognize and respond to PAMPs (as reviewed in Brown & Gordon, 2005) via pattern recognition receptors (PRRs), which recognize and bind to specific groups of PAMPs. PRRs are part of the universal innate immune system found in metazoans (Janeway & Medzhitov, 2002). In arthropods, for example, β-1,3 glucans are recognized by β-1,3 glucan recognition proteins (βGRPs) and gram negative binding proteins (GNBPs) (as reviewed in Brown & Gordon, 2005). βGRPs and GNBPs contain binding domains with significant homology to bacterial β-glucanases, but unlike the bacterial β-glucanases they do not have enzymatic activity (i.e. cannot hydrolyze and break down β-1,3 glucans; Royet, 2004). Instead, most invertebrate βGRPs and the GNBPs first bind to the β-1,3 glucan and then trigger immune responses such as the prophenoloxidase cascade or the production of antimicrobial proteins that are released into the hemolymph (Gobert et al., 2003; Ferrandon et al., 2004).

In other words, while β-1,3 glucan receptors are common in arthropods, active β-1,3 glucanases (henceforth referred to as βGlu) with the ability to hydrolyze and break down β-1,3 glucans are rare in animals (Genta et al., 2003). Plants, however, do
commonly use βGlu’s as a frequent part of their fungal defense (Leubner-Metzger & Meins, 1999). Plants use βGlu’s in two different ways: by either acting directly against the pathogen by breaking down cell walls, or by promoting the release of cell-wall derived materials that act as elicitors of a defense response (as reviewed in Leubner-Metzger & Meins, 1999). Recently βGlu’s have been found in several invertebrates (Bachman, 1996; Genta et al., 2003; Kovalchuk et al., 2006; Pauchet et al., 2008) including one termite species (Bulmer et al., 2009). In the lepidopteran, Helicoverpa armigera, a β-1,3 glucan binding protein with βGlu activity is secreted into the midgut. Whether this βGlu functions in digestion or immunity is not yet clear (Pauchet et al., 2008). If it has an immune role, it could break-down β-1,3 glucans and activate other immune responses (Lamb et al., 1989) or it could function directly against pathogenic fungi or bacteria by breaking down their cell walls (Pauchet et al., 2008).

In the American cockroach, Periplaneta americana, several βGlu’s were isolated including a salivary secreted endo-β-1,3 glucanase, which may play a role in digesting some of the fungus naturally ingested with the roach’s fungal-rich diet (Genta et al., 2003). It is also conceivable that these βGlu’s play a dual role of digestion and pathogen defense, as they are able to hydrolyze fungal cell walls (Genta et al., 2003) and would unlikely discriminate between pathogenic or nonpathogenic fungi. This idea, although recognized by Genta et al. (2003), was only given little consideration and has not been studied empirically.

Most recently, Bulmer et al. (2009) showed that in the termite Nasutitermes corniger, GNBP functions as both a pattern recognition protein and as an effector molecule with βGlu enzymatic activity. Their research suggests that β-glucans are bound
and catalyzed at the intact glucanase region of the GNBP and a separate upstream region binds to LPS. When the βGlu activity of the GNBP was blocked using D-Glucono-1,5-lactone (GDL) the termites experienced significantly higher pathogen related mortality from the entomopathogenic fungus, *Metarhizium anisopliae*, as well as from opportunistic pathogens, which likely benefited from the termites’ suppressed immunity. This study clearly illustrated the role of GNBP, which function as both recognition factors and effector molecules in termite immune defense.

It is unknown if the GNBP of lower termites possess similar functionality to that of the higher termite, *N. corniger*. However, lower termites indeed have βGlu’s (Bulmer et al., 2009), which have been identified and characterized in the lower termite *Mastotermes darwinensis* (Bulmer and Crozier, 2006). One immediate difference between higher and lower termite βGlu’s is that lower termites have multiple active βGlu’s localized in the gut in addition to the two βGlu’s found in the head and gutted body (Bulmer & Rosengaus unpublished data; fig. 1). The nature of these gut-specific βGlu’s is the focus of this chapter.

Lower termites, unlike higher termites, have cellulolytic flagellated protozoa in their hindgut, which have historically been considered important in aiding the breakdown of cellulose for their hosts (Inoue et al., 2000). We have hypothesized that in addition to the nutritional role, these protozoa and/or their associated bacteria are responsible for the synthesis of the additional βGlu’s. Because β-1,3 glucan receptors and βGlu’s are a critical part of the insect immune system (Brown & Gordon, 2005; Gottar, 2006; Bulmer et al., 2009), understanding the origin and function of these glucanases is crucial to a better understanding of termite immunity. Particularly intriguing is the possibility that
lower termites rely on a combination of endogenous and symbiont produced βGlu’s as part of an integrated immune defense strategy. This research, through a series of defaunation and cultivation experiments, clarifies the origin (metazoan vs. symbiotic) of these βGlu’s, which is the first step in elucidating their function.
Materials and Methods

Termite collection

Colonies of the dampwood termite, *Zootermopsis angusticollis*, were collected from the Huddart Park (San Mateo County, CA) and the Redwood East Bay Regional Park in Oakland, CA in July of 2003 and more recently in August 2008. Lab-reared colonies of *Z. angusticollis* were established by pairing male and female alates selected from field collected colonies. Colonies of the eastern subterranean termite, *Reticulitermes flavipes* were collected from Middlesex Fells Reservation, Stoneham MA in April of 2007. All colonies have been maintained in our USDA approved containment room at 25°C inside closed plastic containers and were given water and birch wood as food *ad libitum*.

Defaunation and faunate control procedure

The majority of hindgut termite symbionts are anaerobic (Breznak, 1983). Thus, through the use of an oxygenation treatment, researchers have previously eliminated all of the flagellated protozoan community while leaving a portion of the bacterial community intact without injuring the termite (Cleveland, 1925a; Messer and Lee, 1989). In order to defaunate termites, the oxygenation system (depicted in Ch. 1., fig. 3) was devised. This procedure was adapted from Cleveland (1925b). Nymphs and pseudergates of *Z. angusticollis* were selected from lab-reared or field collected colonies and then placed inside open-ended 15 ml plastic culture tubes. Tubes were lined with a strip of paper towel moistened with 300 µL of sterile water. The tubes were then plugged with moistened cotton-balls and placed into a steel pressure canister (Sure-Shot...
Atomizer©). The canister containing the termites randomly selected for defaunation was next connected to an oxygen tank, which was first flushed with oxygen for 60 seconds to remove residual air. The steel canister was then sealed as oxygen from the tank continued to flow into it, until a pressure of 40 psi was reached. The termites (n=10/tube) remained under high-pressure oxygen for 1 hour and then the canister was depressurized (but never opened). The termites remained under these concentrated oxygen levels (≈97%) for 24 hours, post depressurization. Control faunated termites were subjected to identical manipulations as those of defaunated nestmates with the exception that they were not exposed to oxygen. They were, however, subjected to 40 psi of pressure (pressurized by using a bicycle pump) and also remained in the pressurized steel canister for 1 hour. The canister was then depressurized and the termites remained in the closed container for 24 hours. After treatment, a termite from each group was randomly selected for confirmation of the effectiveness of treatment by visually inspecting (compound light microscope at 400X magnification) the hindgut fluid using a hemocytometer to record the presence or absence of protozoa (Chapter 1, fig. 4).

*Ex vivo symbiont culture techniques*

In order to determine if βGlu’s isolated from the gut tissue were produced by symbionts, the hindgut symbionts were cultured *ex vivo*. The protocol from Coleman (1991) was followed with a few modifications. Adaptations were as follows: the gut symbionts were obtained from the ruptured termite hindgut by using a sterile gel loading pipette tip, rather than a sterile syringe; symbionts were cultured in glass tubes with screw top caps. Capped inoculated culture tubes were incubated at 27°C and sub-cultured
after 2-3 weeks by inoculating 2 ml of the original culture into 10 ml of new culture medium. Cultures were checked periodically using a hemocytometer in order to quantify protozoa numbers. Some cultures were supplemented with antibiotics following the procedure outlined in Colman (1991), in an attempt to create axenic cultures.

**Bradford Assay**

Protein concentrations were quantified using the Bradford method. Following the standard microplate protocol for the Quick Start Bradford Protein Assay (Bio-Rad, USA), 250 µl of Bradford agent was added to 5 µl of protein sample. Bovine serum albumin (Bio-Rad, USA) was used as the protein standard. All protein samples were prepared at three different dilutions and were dispensed into aliquots in triplicate to microplates. The spectrophotometer was set at 595 nm and data were analyzed using the KC4 program.

**Extract Preparations:**

**Defaunated and faunated gut extract preparation**

To test differences in β-1,3 glucanase (βGlu) activity between faunated and defaunated termites, extracts were prepared in the following manner prior to chromogenic electrophoresis. Defaunated and faunated termites were cold immobilized. After immobilization, the termite gut was extracted by using fine tipped tweezers to remove the gut via the anus. The whole gut was then placed into a Biomasher® filter (pore size 80-145 µm; Cartagen), and the corresponding gutted body was placed into a second Biomasher®. Guts from two termites were pooled in one tube and the corresponding
gutted bodies were pooled in a separate tube. The contents of the tubes were ground with a pestle and then centrifuged (4°C; 14,000 X g; 1 min). Some extracts were then standardized using the Bradford assay (see above). All extracts were then used for chromogenic electrophoresis on Carboxymethyl Curdlan Remazol Brilliant Blue (CM-curdlan-RBB) gels as described below.

**Preparation of foregut, midgut, and hindgut extracts**

In order to localize the region of the alimentary tract where βGlu’s originate, termite guts were divided into the foregut, midgut, and hindgut sections and prepared as follows for chromogenic gel electrophoresis. Termite guts were removed using previously described methods and dissected into foregut, midgut and hindgut (Chapter 1; fig. 1) under a dissecting microscope (Nikon SMZ800). Gut sections were placed into separate Biomashers®, centrifuged (4°C; 14,000 X g; 1 min). Protein was then quantified using the Bradford assay and then standardized to avoid protein differences resulting from the various sizes of the gut sections. These extracts were then used for electrophoresis on CM-curdlan RBB gels as described below.

**Separation of ex vivo symbionts from culture fluid and preparation of extract**

In order to test if *ex vivo* cultured symbiont extracts exhibited βGlu activity, symbionts were first separated from the 12 day culture fluid and then concentrated prior to gel electrophoreses. Symbionts were separated from the culture fluid following a modified protocol from Yamin (1978). Three to four ml of culture fluid were layered over 6 ml of chilled 20% Ficoll 400 (Sigma Chemical Co.) solution in a 10 ml culture
tube. Samples were centrifuged (4°C; 900 X g; 20 min). Protozoa consisting of mostly *Trichomitopsis termopsidis* (Chapter 1; fig. 2) along with associated bacteria were collected from the interface. To ensure a clean sample, the interface fluid was re-centrifuged (4°C; 8000 X g; 30 min) and the supernatant removed and discarded. The remaining pellet was resuspended in 250 µl of Trager U solution (Trager, 1934) and again centrifuged (4°C; 8000 X g; 10 min). The supernatant was again discarded and the remaining pellet was resuspended a final time with 50 µl of Trager U. Ten µl of the final suspension was examined under the microscope (Nikon Eclipse E400, 400X magnification) to confirm presence of protozoa. These samples were then used for chromogenic electrophoresis on CM-curdlan-RBB gels as described below.

*Separation and extract preparation of symbiont fractions*

To examine if there were differences in βGlu activity between the lumen bacteria and the protist-bacteria consortium, termite guts were separated based on density (Morgavi et al., 1994) and then run on a CM-curdlan-RBB gel. Two termite guts were extracted and their contents (but not the gut tissue itself) were transferred to 1 ml of sterile Trager U (Trager 1934) solution. One of the emptied guts was gently washed in sterile Trager U and rinsed with a syringe in order to test β-1,3 glucanase activity of the gut tissue alone. The gut contents were centrifuged at a low speed (4°C; 550 X g; 5 min) to pellet down the protozoa and their associated bacteria, while leaving the other bacteria in suspension. The supernatant was extracted and placed into a separate tube and centrifuged at a high speed (4°C; 20,800 X g; 30 min) to collect the bacterial pellet. The supernatant from the second spin was saved as a cell-free fraction. Native sample buffer
(Bio-Rad) was added (2:1 ratio) to the protozoa/bacteria pellet, bacterial pellet, and the cell free fraction prior to loading in the CM-curdlan-RBB gel.

**Visualization of β-1,3 glucanases using chromogenic CM-curdlan-RBB gels**

In order to visualize the βGlu’s, termite extracts were electrophoresed on Carboxymethyl Curdlan Remazol Brilliant Blue (Loewe Biochemica) gels (CM-curdlan-RBB). The CM-curdlan-RBB gels were prepared following the method from Kalix and Buchenauer (1995). Native Sample Buffer dye (2:1 dilution) was added to each extract sample to facilitate gel loading (Bulmer et al., 2009). The CM-curdlan-RBB gels contain β-1,3 glucans that are bound to the Remazol brilliant blue dye. After migration through the gel, βGlu’s digest the β-1,3 glucans and the dye is then released and subsequently washed off by sodium acetate (NaAc), resulting in clearing zones that indicate the relative number and sizes of the βGlu’s. The gels were loaded with the purified termite tissue extracts, electrophoresed at 50 volts for twenty minutes, and then run at 150 volts until completion (approx. 60 minutes). The gel was then incubated with a 100 mM NaAc buffer (pH = 5.0) for 18-20 hours on a shaking platform (50 RPM). Some gels were also incubated with a 100mM D-Glucono-1,5-lactone (GDL) a specific inhibitor of β-1,3 glucanases. For the GDL assay, half of each sample was loaded into lanes on separate sides of the gel. After the gel was run to completion one half of the gel, with a full set of samples, was incubated in a developing buffer with 100 mM NaAc and the other half with 100 mM GDL buffer. All gels were rinsed with deionized water after incubation. Gels were photographed using a Kodak Gel Logic 10 camera.
Results

Our data demonstrated that upon removal of symbionts with hyperbaric oxygen, a clear loss of βGlu activity was observed. Additionally, symbionts cultured *ex-vivo* for 12 days exhibited strong βGlu activity. By culturing the hindgut symbionts *ex-vivo*, we ensured no βGlu contamination from any of the termite tissue, thus corroborating that the multiple βGlu’s in our 12 day old cultures were of protozoan origin. The results of each experiment are discussed, in turn, below.

Detection of β-1,3 glucanases in faunated and defaunated termite extracts

Crude gut extracts of the defaunated (i.e. oxygenated) *Z. angusticollis* (fig. 2 & 3) and *R. flavipes* (fig. 4) both exhibited a loss of βGlu activity compared to the guts of their faunated counterparts. Our results therefore indicate that the presence of symbiont-produced βGlu’s in hindguts could be widespread throughout the lower termite species (fig. 1).

Extracts of *Z. angusticollis* faunated guts (fig. 2; lane 3) showed several clearing zones resulting from βGlu activity. The defaunated guts, on the other hand, showed a loss of βGlu activity (fig. 2; lane 1). In other words, loss of a high fraction of the symbiont community coincided with loss of βGlu activity. The faunated and defaunated bodies showed only two βGlu clearing zones (fig. 2; lanes 2 & 4). In fact, these same two bands were present in all *Z. angusticollis* tissues and similar bands were found in *Reticulitermes flavipes*. It is likely that these two ubiquitous clearing zones represent potentially endogenous βGlu’s. These “endogenous” glucanases are likely GNBPs produced by salivary glands, as both primitive and advanced termites appear to produce
GNBPs with βGlu activity (Bulmer et al., 2009). However, further characterization would be needed to confirm this hypothesis. Nearly identical clearing zones were seen each time these gels were run. To ensure that this phenomenon was unrelated to the quantity of protein present in our samples, the same four treatments of Z. angusticollis were run with a standardized protein quantity (9mg/ml) (fig. 3). The results were similar with a few exceptions. The defaunated gut (fig. 3; lane 3) showed three areas of β-1,3 glucanase activity rather than the two seen in non-standardized gels (fig. 2). The protein standardized defaunated gut still had fewer clearing zones than its faunated counterpart (fig. 3; lane 1 & 3), which had at least six areas of βGlu activity. Therefore we are confident that the presence (or absence) of clearing zones are not artifacts of overall protein content in our samples.

A similar pattern was found in Reticulitermes flavipes (fig. 4). While the fully faunated gut exhibited multiple zones of βGlu activity (fig. 4, lane 2), the defaunated gut had only two clearing zones (fig. 4; lanes 4). The defaunated and faunated R. flavipes bodies were also similar in that they showed the two possibly endogenous βGlu’s that were also identified in the defaunated gut (fig. 4; lanes 1 & 3). Cryptotermes, another phylogenetically intermediate lower termite appears to also have multiple clearing zones in the faunated gut and two in the head/body (Bulmer et al., 2009). This is the first multi-species comparative approach to look at the coinciding loss of both symbionts and βGlu activity. The patterns found here suggest that other lower termites may have two potentially endogenous βGlu’s, and that the additional βGlu activity seen in gut contents is of symbiont origin.
Detection of β-1,3 glucanases in termite gut sections

In addition to determining if βGlu activity was connected to the presence of symbionts, we also wanted to determine the specific region of the alimentary tract associated with this activity. We predicted that if βGlu’s originated from protozoa and their associated bacteria, we should only detect specific clearing zones in the hindgut region that are not found in other sections of the digestive system. Termites have bacteria throughout their alimentary tract while protozoa are primarily located in hindgut paunch (Brune, 2006). Therefore separating the gut into sections allowed us to narrow down the source of the symbiotic βGlu’s.

Protein standardized sections of Z. angusticollis guts (1.5 mg/ml) showed distinctly different regions of β-1,3 glucanase activity (fig. 5). The foregut and midgut showed clearing zones that correspond to the potentially endogenous bands found in the gutted body (fig. 5; lane 1 & 2), while the dissected hindgut section showed a clear similarity to the bands seen in the intact Z. angusticollis gut (fig. 5; lane 3).

Inhibition of β-1,3 glucanases by D-Glucono-1,5-lactone (GDL)

As GDL inhibited the βGlu activity of GNBP-2 in the higher termite, Nasutitermes corniger (Bulmer et al., 2009), we tested whether GDL could similarly inhibit the βGlu’s of Z. angusticollis. Our results show that all βGlu’s in Z. angusticollis were inhibited by incubation with 100mM GDL (fig. 6A & 6B). The half incubated with NaAc showed the expected clearing zones (fig. 6B), whereas the half incubated with GDL showed no β-1,3 glucanase activity (fig. 6A).
Detection of β-1,3 glucanases in fractions of termite gut fluid

In order to further characterize which members of the symbiont hindgut community are producing βGlu, a centrifugal separation of the larger protist and smaller bacterial community was performed. After separation, of gut fluid, into three fractions, each fraction was examined under a microscope at 400X magnification. The pellet from the initial low-speed spin contained protists and associated bacteria (protist/bacteria fraction). The pellet from the second high speed spin, contained only bacteria and no protists (bacterial fraction), while the supernatant (cell-free fraction) did not contain any visible cells. βGlu activity of the three fractions (protist/bacteria, bacteria, and cell-free) was distinct (fig. 7). When the βGlu’s of the fractions were compared to those of a termite gutted body, an intact termite gut, and a washed termite gut (fig. 7), the washed gut had decreased βGlu activity compared to the intact unwashed gut or the protist/bacteria fraction. The bacterial fraction showed less βGlu activity than the protist/bacteria fraction. The cell-free fluid showed no detectable β-1,3 glucanase activity (fig. 7). These results, together with those of the different regions of the termites’ digestive tract indicate that the majority of the glucanases found in the termite are originating from the hindgut region and are likely produced by the protists and their associated bacteria rather than the lumen bacteria.

*Termite ex vivo symbiont cultures*

To test if termite symbionts maintain βGlu activity independent of their termite host, several symbiont cultures were established. Symbiont cultures were visually checked every 3-5 days for symbiont viability. The symbionts remained viable in
cultures for up to two months. Typically after two weeks the dominant protozoa present was *Trichomitopsis termopsidis* (Chapter 1; fig. 2). This larger protozoan is one of the few to have been cultured axenically and is known to digest cellulose (Yamin, 1978; Yamin & Trager, 1979). In our cultures, bacteria were always visible in the fluid along with the protists. Attempts were made to remove the bacteria with antibiotics, however this always resulted in protist death. This is not surprising since termite protists are known for being extremely difficult to culture (Inoue et al., 2000; Slaytor, 2000; Brune, 2006; Breznak, personal communication) and only a handful of studies have successfully cultured them axenically (Yamin, 1978, 1980; Odelson & Breznak, 1985).

Detection of β-1,3 glucanases in termite symbiont cultures

*Ex vivo* cultured symbionts exhibited identical βGlu activity to gut fluid obtained directly from the termite (fig. 8 & 9). The *ex vivo* symbionts were cultured for twelve days prior to the assay in order to minimize the possibility of contamination with termite derived βGlu’s. Cultured symbionts were then electrophoresed on CM-curdlan-RBB gels. The gels showed clear bands of β-1,3 glucanase activity that were similar to the fully faunated gut (fig. 8). Microscopic examination of the culture used for this assay showed predominantly *Trichomitopsis termopsidis* and *Trichonympha* spp. (Chapter 1; fig. 2). When a protein standardized gel (2.3 mg/ml) was run, microscopic examination of that culture tube showed *T. termopsidis* and only a few *Hexamastix* spp. (Chapter 1; fig. 2). The standardized gel exhibited areas of βGlu activity similar to that of a faunated gut (fig. 9). As a control, culture fluid without symbionts was also electrophoresed and it did not
exhibit any glucanase activity (fig. 9). Our results therefore identify three genera of protozoa and their associated bacteria as very strong candidates for βGlu production.
Discussion

Our data clearly support a hindgut symbiont origin of βGlu’s in Z. angusticollis as well as in R. flavipes. Although we have only sampled a few species out of the total 2600 described Isopteran species (Kambhampati & Eggleton, 2000), the limited current survey (fig. 1) points toward a universal presence of two potentially endogenous βGlu’s and multiple symbiont-derived βGlu’s. This pattern may be widespread throughout the lower termite groups, but clearly, further analyses using a wide range of termite species are required. Additionally, it would be of great interest to tests if the woodroach Cryptocercus punctulatus, the likely proto-termite ancestor (Lo et al., 2000), also exhibits similar tissue-associated and gut-derived βGlu’s.

Origin of β-1,3 glucanases in Z. angusticollis

Cleveland’s classic defaunation experiments found that termites, defaunated via oxygen, died of starvation within 3 to 4 weeks and he thus concluded that termites were unable to digest cellulose without the help of their flagellated protozoa (Cleveland, 1925a). At the time of these early defaunation experiments, relatively little was known of the termite gut symbionts. Cleveland assumed that defaunation via oxygen left the bacterial population intact. Yet, more recent studies indicate that this is not the case (Slaytor, 2000). Many bacteria are closely associated with protists: they are attached to or live within the protist cells (reviewed in Breznak, 2000), making it impossible to eliminate the protists while leaving the bacterial community undisturbed. The difficulty of separating and culturing individual species of the termite hindgut microbiota is one of
the biggest challenges in assigning specific roles to members of these diverse microbial consortia (Breznak, 2000). While we have not yet completely identified the member(s) of this assemblage responsible for the βGlu synthesis, our results point to at least three distinct protozoa/bacteria candidates and open up the possibility of an additional role besides nutrition. The Z. angusticollis gut consortium not only produces cellulases for the digestion of cellulose, but it also appears to secrete a variety of βGlu’s that may help in the breakdown of ingested fungi and could perhaps be involved in providing protection to the host against fungal invasion (see Chapter 1).

Removal of the anaerobic symbionts resulted in a clear and consistent loss of the hindgut specific βGlu’s, but did not affect the two βGlu’s associated with the termite body and gut tissue (fig. 2, 3, & 4). These data combined with the occurrence of βGlu’s identified in symbiont cultures (fig. 8 & 9) provides evidence for symbiont produced βGlu’s, which are separate from the potentially endogenous βGlu’s. The roach, P. americana has salivary βGlu’s (Genta et al., 2003). It is possible that the βGlu’s consistently associated with Z. angusticollis and R. flavipes tissue are salivary βGlu’s, as well. Given the phylogenetic relationship between roaches and termites (Nalepa & Bandi, 2000) and the apparent widespread presence of tissue associated βGlu’s in termites (fig. 1), it seems likely that endogenous βGlu’s were already present in roach-like ancestors of termites. These salivary glucanases of the proto-termite could have been an important pre-adaptation in that they may have facilitated the breakdown of fungi colonizing their nests, thus providing protection against pathogenic fungi.

Within the termite gut, different sections of the gut house different symbiont communities (Breznak, 2000; Brune, 2006). In order to localize the region with the
highest βGlu activity, the gut was dissected into foregut, midgut, and hindgut. The foregut and midgut both showed glucanase activity similar to the gutted termite body (fig. 5). If these tissue associated glucanases are produced by the salivary gland they would be secreted into the buccal cavity (Noirot, 1969) and washed downstream into the rest of the alimentary tract. That would then explain their stronger concentration in the foregut, which is closer to the source of secretion. If instead these βGlu’s are actually secreted by the gut tissue, then the gut, even when washed, should have maintained strong activity in these regions, which it did not (fig. 7). This further supports our salivary origin hypothesis of these potentially endogenous βGlu’s. The hindgut section, on the other hand, possessed the most diverse βGlu activity (fig. 5), which was similar to the activity found in the symbiont cultures (fig. 8 & 9) and in expressed liquid termite feces (Bulmer & Rosengaus, unpublished data; fig. 12). In contrast to our study, Genta et al. (2003) did not find any βGlu’s in the hindgut of roaches that were distinct from those found in the foregut or the midgut. In Z. angusticollis, these multiple active βGlu’s are synthesized in the hindgut and are not likely to have been “washed” into the lower digestive tract, as they are not present in upper sections of the gut.

While these data strongly indicate a symbiont-origin of βGlu’s in the hindgut region of the termite’s digestive tract, discerning precisely which member(s) are responsible remains a problem to be tackled. In an attempt to eliminate bacterial symbionts several antibiotic incubations of the protist cultures were undertaken, but all resulted in the death of the protists. As an alternative to bacterial elimination, a separation of lumen bacteria from protists and their associated bacteria was performed. Bacteria found swimming freely in the lumen of the termite gut differ from the bacteria
attached to and within the protists (Brune, 2006). Our results show significantly more βGlu activity associated with the protists and their bacteria than with the free-living lumen bacteria (fig. 7). The flagellated protists and their intimately associated bacteria would be a good focal point for further molecular work to identify the source and characterize these βGlu’s.

A culture-independent technique was successfully applied by Inoue et al. (2005) to characterize a cellulase gene in a symbiotic termite protist. They cloned the β-1,4 glucanase gene (i.e. cellulase gene) from a cDNA library constructed from a mixed population of hindgut protists in the lower termite Coptotermes formosanus. By screening the resulting cDNA library for cellulolytic activity using a Congo Red technique, they were able to distinguish cellulose activity. Then by application of gene specific primers and whole-cell in situ hybridization they identified the protist Spirotrichonympha leidyi as the source of the cellulase gene. Similar molecular techniques may provide insights into the origin of the hindgut symbiont derived βGlu’s.

*Role of β-1,3 glucanases in Z. angusticollis*

Termites have recently been placed in a monophyletic group together with the roach, *P. americana*, and wood roaches of the genus *Cryptocercus*, within the Dictyopteran clade (mantids, cockroaches, and termites; Lo et al., 2000). The ancestor of the entire Dictyopteran clade was likely an omnivorous detritus feeder emerging during the Jurassic (200-150 million years ago; Lo et al., 2000). It is hypothesized that mantids diverged early in the Dictyopteran lineage followed by a rapid radiation within cockroaches. The lineage leading to *P. americana*, *Cryptocercus* and termites adapted
from detritivore feeding habits to wood feeding (Lo et al., 2000). The acquisition of protozoa with β-glucanases in the roach-like ancestor likely supplemented endogenous glucanases and would have been key in this transition (Lo et al. 2000). While the origin of animal β-glucanases remains under debate (Watanabe & Tokuda, 2001), there is some evidence of horizontal gene transfer between species (Garcia-Vallvé et al., 1991; Guiseppi et al., 1991; Yan et al., 1998). The possibility then exists that termites acquired at least two endogenous βGlu’s from their symbionts via horizontal gene transfer.

The added access to food sources that βGlu’s could provide is consistent with a digestive role hypothesis. In P. americana, two βGlu’s (LAM and LIC 1), both of which are found in the salivary glands, lyse fungal cell walls and may allow the roach to maximize available nutrients (Genta et al. 2003). It is possible that the two putatively endogenous βGlu’s associated with all Z. angusticollis tissues function in a similar digestive role. The phylogenetically primitive, Z. angusticollis, like P. americana, do forage on fungal-rich food sources (Noirot & Noirot- Timothée, 1969; Genta et al., 2003) and it is conceivable that they too would benefit nutritionally from access to fungal cell components in addition to cellulose.

However, an alternate or additional hypothesis to a digestive function of the gut βGlu’s is that of protection against pathogens. This is a hypothesis that has been put forth to explain the presence of active βGlu’s in the foreguts and midguts of both P. americana and H. armigera (Genta et al., 2003; Pauchet et al., 2008), but not empirically tested.

In addition to the two potentially endogenous βGlu’s associated with the termite tissue, we also see the presence of multiple symbiont produced βGlu’s. While these
could have a digestive function, they could also provide symbiont-mediated pathogen protection to the insect host. There are several insect examples in which the presence of symbionts increases the host’s resistance to predation and also against pathogenic agents (reviewed in Haine, 2007; Lombardo, 2008; also see Chapter 1). For example the beetle, *Paederus riparius*, is protected from predation by a toxin produced by its bacterial endosymbionts (Kellner, 1999). Another invertebrate example includes the shrimp *Palaemon macrodactylus* whose embryos gain resistance to fungal infection by metabolites produced from *Alteromonas* spp. bacteria (Gil-Turnes et al., 1989). Pea aphids are also known to receive protection against both fungal parasites and parasitoid wasps due to their facultative bacterial symbionts (Oliver et al., 2002; Scarborough et al., 2005).

In a similar manner, the symbiont-derived βGlu’s of termites could function in pathogen defense. One method by which the βGlu’s could provide protection to the host is by directly hydrolyzing the cell wall of microbes and consequently killing the pathogenic cell. This is one mode of action by which some plant βGlu’s provide protection against infection (Leubner-Metzger & Meins, 1999). Alternatively (or additionally), hindgut βGlu’s could work synergistically with other metabolites by “punching holes” in fungal cell walls and allowing access to fungicidal agents as suggested by Bulmer et al. (2009). In a comparable manner, tobacco βGlu’s work in conjunction with chitinases to effectively inhibit the *in vitro* growth of the plant fungal pathogen *Fusarium solani* (Leubner-Metzger & Meins, 1999). A third possibility is that symbiont-derived βGlu’s release β-1,3 glucan substrates, which are known to be immune elicitors (Gobert et al., 2003; Ferrandon et al., 2004). This is illustrated by Soybean
βGlu’s, which release β-1,3 glucans from fungal cell walls that then induce an accumulation of phytoalexin glyceollin, a plant antibiotic (Sharp et al., 1984).

Our in vivo studies on Z. angusticollis have shown susceptibility to pathogens increases when a large portion of its hindgut community is removed via oxygen (Chapter 1), which we now know, also eliminates all but two βGlu’s. In vivo and in vitro studies on the termite gut indicate that it is hostile to fungi (Yanagawa & Shimizu, 2007; Bulmer et al., 2009; Chouvenc et al., 2009). Conidia of M. anisopliae are unable to germinate in the alimentary tracts of the termite Coptotermes formosanus (Yanagawa & Shimizu, 2007). Furthermore, the guts of R. flavipes have lingering fungal resistance post-mortem, as they are the last tissue to be invaded by M. anisopliae (Chouvenc et al., 2009). The combination of termite gut fungal resistance and increased mortality of defaunated termites leads to the question of whether termite symbionts are producing antimicrobials, which benefit the termite host by enhancing its resistance to mycosis. The symbiont βGlu’s are prime candidates for this antimicrobial action. Some βGlu’s are known to possess fungistatic properties against fungal pathogens both in plants (Leubner-Metzger & Meins, 1999) and animals (Bulmer et al., 2009). Possibly the βGlu’s of symbiont origin contribute to the fungistatic nature of the termite gut.

As termites are eusocial insects (Wilson, 1971) these data must also be examined in the context of their sociality. The prime influences favoring the evolution of social behaviors include increased predator defense and foraging efficiency as well as improved ability to locate mates and to modify the environment (Wilson, 1975). However, with the benefits of group living there are also significant drawbacks, among which, increased exposure to parasites is one (Schmid-Hempel, 1998). Despite the increased likelihood of
parasite transmission amongst closely related individuals (Schmid-Hempel, 1998), there is much evidence that termites and other eusocial insects exhibit group behaviors that mitigate the effect of disease (Hughes et al., 2002; Traniello et al., 2002; Rosengaus et al., 2004 and references therein; Ugelvig & Cremer, 2007). One such group behavior found in honeybees is inducing a “fever” in the hive, in order to facilitate defense against pathogens (Starks et al., 2000). A group behavior exhibited by termites to mitigate the effects of fungal pathogens is to remove the conidia from the cuticles of nestmates, which significantly decreases the group’s susceptibility to disease (Rosengaus et al., 1998a; Yanagawa & Shimizu 2007, Yanagawa et al., 2008). A third mechanism of group-mediated pathogen defense, also seen in termites, includes alarm signals that communicate information to nestmates about the presence of pathogens (Rosengaus et al., 1999b).

Further support for the idea of group-mediated pathogen defense has been reported in naïve termites and ants (not directly exposed to a pathogen), which apparently receive immune benefits via association with their nestmates that had been exposed to non-lethal doses of pathogens (Rosengaus et al., 2002; Ugelvig & Cremer, 2007). One hypothesis for the increase in pathogen defense seen in naïve termites associated with immunized nestmates is that immune factors are transferred via trophallaxis (Traniello et. al., 2002; Ugelvig & Cremer, 2007). Social insects engage in the transfer of secretions via both oral trophallaxis and proctodeal feeding amongst nestmates (Wilson, 1971). While immune benefits have been hypothesized, trophallaxis is also a potentially efficient pathway, which could be exploited by a parasite for its own transmission (Schmid-Hempel, 1998). Given the exploitability of this pathway, there is a potential that a
counter-mechanism to avoid that exploitation has evolved. As we have shown that hindgut symbionts produce βGlu’s, and termites are known to transfer their symbionts during proctodeal trophallaxis (Andrew, 1930), these βGlu’s could possibly function as added pathogen defense during proctodeal feedings. This is even more convincing considering that βGlu activity is found in the liquid feces (Bulmer & Rosengaus unpublished; fig. 10), which are nearly identical to those produced by the symbionts (fig. 2). Additionally, termite solid feces, which are widely used in the Isoptera for the construction of nests, are highly antifungal (Rosengaus et al., 1998b). Possibly the antifungal activity found in these feces is due to the symbiont produced βGlu’s. If βGlu’s are directly shared among nestmates during proctodeal feeding, these symbiont-derived βGlu’s could have a dual function (i.e. nutrition and disease resistance) for both the individual and the colony. Thus, contrary to expectation, sociality may reduce rather than increase, disease susceptibility at both the individual level and the colony level.
Figure 1: β-1,3 glucanase activity in three termite species. Shown here is β-1,3 glucanase activity from Z. angusticollis (lane 1 & 2), R. flavipes (lane 3 & 4) and N. corniger (lane 5 & 6; gel from Bulmer & Rosengaus, unpublished). The Z. angusticollis gut (lane 2) and R. flavipes gut (lane 4) have visible β-1,3 glucanases not present in gutted bodies (lanes 1 & 3, respectively). There appears to be no difference in glucanase activity between the gutted body and the gut in N. corniger (lane 5 & 6).
Figure 2: CM-Curdlan-RBB gel of faunated and defaunated crude Z. angusticollis extracts. Crude extracts of two termites were pooled before loading into lanes. Crude extracts of termites were loaded in the following order: defaunated (DF) gut (lane 1), defaunated gutted body (lane 2), faunated control (FC) gut (lane 3) and faunated control gutted body (lane 4).
Figure 3: CM-Curdlan-RBB gel of protein standardized faunated and defaunated crude Z. angusticollis extracts. Crude protein standardized (9mg/ml) extracts of two termites were pooled before loading into the following order: faunated pressure control (FC) gut (lane 1), faunated pressure control body (lane 2); defaunated (DF) gut (lane 3), defaunated body (lane 4). The faunated pressure control termite gut (lane 1) indicates that pressure treatment alone does not have an effect on β-1,3 glucanase activity. Pressure with the addition of oxygen (i.e. defaunation) does effect the β-1,3 glucanase activity seen in the gut (lane 3).
Figure 4: CM-Curdlan-RBB gel of faunated and defaunated *R. flavipes* crude termite extracts. Crude extracts of five termites were pooled before loading into lanes. Crude extracts of *R. flavipes* workers were loaded in the following order: faunate control (FC) gutted body (lane 1), faunate control gut (lane 2), defaunated (DF) gutted body (lane 3) and defaunated gut (lane 4).
Figure 5: CM-Curdlan-RBB gel of foregut, midgut, and hindgut extracts from Z. angusticollis. Gut sections of several termites were pooled and protein standardized (1.5mg/ml) before loading into lanes. Gut extracts of gut sections were loaded in the following order: foregut (FG) (lane 1), midgut (MG) (lane 2), and hindgut (HG) (lane 3).
Figure 6: CM-Curdlan-RBB gel of faunated and defaunated crude Z. angusticollis extracts incubated with and without 100 mM GDL. Crude extracts of two termites were pooled before loading into lanes. Crude protein standardized (14mg/mL) extracts of termites were loaded in the following lanes in the same order for both gels: defaunated (DF) termite gut (lane 1), defaunated gutted body (lane 2), faunated control (FC) gut (lane 3) and faunated control gutted body (lane 4). After electrophoresis, gels were incubated in sodium acetate buffer (pH=5.0) supplemented with 100 mM GDL (A.) or with sodium acetate buffer alone (B.).
Figure 7: CM-Curdlan-RBB gel of termite symbiont separations. Z. angusticollis crude extracts along with centrifugal separations of termite symbionts were loaded in the following order: faunated gutted termite body (FB; lane 1), faunated termite gut (FG; lane 2), washed termite gut (WG; lane 3), termite protists and protist bacteria fraction (PBF; sample divided into lanes 4 & 5), lumen bacterial pellet (BP; sample divided into lanes 6 & 7), cell free fraction (CF; lane 8). All symbiont fractions were examined microscopically for presence of bacteria and/or protozoa. The lumen bacteria fractions were determined to be protist free, while the mainly protozoa fraction still contained visible bacteria.
Figure 8: CM-Curdlan-RBB gel of faunated Z. angusticollis extracts and Z. angusticollis 12 day old symbiont cultures. Crude extracts of termites and extracts of symbiont cultures were run on the following lanes: faunate control (FC) gutted body (lane 1), faunate control gut (lane 2), extracts ex vivo cultured symbionts (lane 4 & 6).
Figure 9: CM-Curdlan-RBB gel of protein standardized faunated *Z. angusticollis* extracts and termite symbiont cultures. Crude protein standardized (2.3 mg/ml) extracts of termites and extracts of symbiont cultures were run on the following lanes: faunate control (FC) gutted body (lane 1), faunate control gut (lane 2), extract of *ex vivo* cultured symbionts (lane 3), control culture fluid (lane 4).
Figure 10: CM-Curdlan-RBB gel of Z. angusticollis gutted body, gut and liquid feces. Extracts of gutted termite bodies (lanes 1 & 4) show β-1,3 glucanase of potential termite origin. Extracts of whole guts (lanes 2 & 5) show β-1,3 glucanase of symbiont origin. Expressed fresh liquid feces of Z. angusticollis (lanes 3 & 6) show β-1,3 glucanase activity identical to that of the gut.
Chapter 3

*In vitro* effects of *Zootermopsis angusticollis* hindgut symbionts upon the viability of the entomopathogenic fungus, *Metarhizium anisopliae*. 
Introduction

Dampwood termites, *Zootermopsis angusticollis* live at high densities under humid and warm microclimatic nest conditions, all of which favor the growth of potential pathogens (Sands, 1969; Rosengaus & Traniello, 1997; Rosengaus et al., 2003). One such pathogen, the entomopathogenic fungus *Metarhizium anisopliae*, has received a lot of attention as a potential biological control agent (Milner & Staples, 1996; Culliney & Grace, 2000). However, despite its potential, *M. anisopliae* has had limited success as a termite control agent in the field (Rath, 2000). One possible reason for the failure of *M. anisopliae* as a biological control agent is that termites exhibit multiple defenses against disease-causing microorganisms. Within the behavioral defenses of termites, the upregulation of mutual grooming (using their mouthparts) results in the removal of fungal conidia off the cuticles of nestmates (Kramm & West, 1982; Rosengaus et al., 1998a; Yanagawa & Shimizu, 2005; Yanagawa et al., 2008). Although the subsequent ingestion of conidia would seem to put the groomer at an increased risk for fungal infection, several studies have found that passage through the gut inhibits conidia germination (Kramm & West, 1982; Boucias et al., 1996; Rosengaus et al., 1998a; Yanagawa & Shimizu, 2005). The termite gut appears to possess strong antifungal properties that render the conidia inviable. Chouvenc et al. (2009) found that conidia did not germinate within the gut of the eastern subterranean termite, *Reticulitermes flavipes*. Most strikingly, this effect remained post-mortem: termites succumbing to mycosis were examined two days post-mortem and while generalized fungal growth was observed throughout the body cavity, none was observed in the gut. It has been proposed that the fungistatic activity of the termite gut may be due to multiple biochemicals of unknown
origin (Chouvenc et al., 2009). One such compound isolated from *Reticulitermes* spp. is norharmane (Siderhurst et al., 2005; Chouvenc et al., 2008), which is thought to be produced by endosymbionts, most likely Actinomycetes (Siderhurst et al., 2005). While norharmane is toxic to several organisms (Oda et al., 1988; Quetin-Leclerc et al., 1995; Rivas et al., 1999), *in vitro* tests using termite physiological concentrations showed that it had limited effect on mycelial growth of *M. anisopliae* (Chouvenc et al., 2008). Thus, other factors must be involved to account for the full antifungal effect of termite guts.

As termite guts house a diverse consortium of prokaryotic and eukaryotic symbionts (reviewed by Inoue et al., 2000; Breznak, 2000) and there are numerous examples of symbiont-mediated pathogen defenses in invertebrates (reviewed in Chapter 1), we hypothesize that members of the gut community synthesize antifungal metabolites that help protect the host. Our previous research (see Chapter 1) showed that termites, which have undergone defaunation via an oxygenation treatment, experienced significantly higher mortality following exposure to *M. anisopliae* conidia relative to termites whose gut communities were left intact. This indicates that mutualistic gut symbionts are an important part of host resistance.

We have identified a novel alternative metabolite to norharmane, which may be partly responsible for the fungistatic nature of the termite guts. The hindgut mutualistic symbionts of *Z. angusticollis* produce β-1,3 glucanases (βGlu’s; see Chapter 2). βGlu’s are enzymes that are capable of breaking the β-1,3 glycosidic linkages of β-1,3 glucans and are known to provide pathogen protection in many plants (Leubner-Metzger & Meins, 1999) and some animals (Bulmer et al., 2009). Recently, Bulmer et al. (2009) found that a gram negative binding protein (GNBP) in the higher termite *Nasutitermes*
corniger has βGlu enzymatic activity. The dual function of N. corniger’s GNBP (as a pathogen recognition receptor and as an effector molecule) is unusual given that most GNBPs in other organisms only function as pathogen receptors. Because β-1,3 glucans are common cell wall components of pathogens (Pitson et al., 1993; Brown & Gordon, 2005), their recognition and breakdown by this GNBP with βGlu activity is a critical component of N. corniger’s pathogen defense system (Bulmer et al., 2009).

The purpose of the present study is to identify if the gut of Z. angusticollis has in vitro fungistatic activity against M. anisopliae and if that activity is related to the presence of symbionts and symbiont-produced βGlu’s. This question was explored by incubating tissue extract of normally faunated and defaunated Z. angusticollis with M. anisopliae conidia. We predicted that if termites have symbiont-mediated protection against disease, then conidia incubated with extracts of faunated guts should have lower viability than conidia incubated with extracts from defaunated guts. Subsequent studies attempted to establish whether the βGlu’s play a role in the antifungal properties of the guts by adding a glucanase inhibitor, D-Glucono1,5-lactone (GDL) to the tissue extracts. If βGlu has a fungistatic (or fungicidal) effect on conidia, then blocking the βGlu’s active sites should enhance fungal viability relative to non-blocked βGlu’s. Specifically, conidia viability should be rescued when GDL blocks the βGlu’s active sites.
Materials and Methods

Termite collection

Colonies of the dampwood termite, *Zootermopsis angusticollis*, were collected from the Huddart Park (San Mateo County, Ca) and the Redwood East Bay Regional Park (Oakland, CA) in July of 2003 and more recently in August 2008. Lab-reared colonies were established by pairing male and female alates selected from field-collected colonies. All colonies have been maintained in our USDA approved containment room at 25°C inside closed plastic containers and are given water and birch wood as food *ad libitum*.

Defaunation procedure

The majority of hindgut termite symbionts are anaerobic (Breznak, 1983). Thus, through the use of an oxygenation treatment, researchers have previously eliminated all of the flagellated protozoan community while leaving a portion of the bacterial community intact without injuring the termite (Cleveland, 1925a; Messer and Lee, 1989). In order to defaunate termites, we devised the oxygenation system depicted in Chapter 1 (Fig. 3). This procedure was adapted from Cleveland (1925b). Nymphs and psuedergates of *Z. angusticollis* were randomly selected from field-collected or lab-reared colonies and then placed inside open-ended 15 ml plastic culture tubes. Tubes were lined with a strip of paper towel moistened with 300 µl of sterile water. The tubes were then plugged with moistened cotton-balls and placed into a steel pressure canister (Sure-Shot Atomizer©). The canister containing the termites selected for defaunation was subsequently connected to an oxygen tank, which was first flushed with oxygen for 60 seconds to remove residual
The steel canister was then sealed as oxygen from the tank continued to flow into it, until a pressure of 40 psi was reached. The termites (n=10/tube) remained under high-pressure oxygen for 1 hour and then the canister was depressurized (but not opened). The termites remained under these concentrated oxygen levels (≈97%) for 24 hours, post depressurization. Control faunated termites were subjected to identical manipulations as those of defaunated nestmates with the exception that they were not exposed to oxygen. They were, however, subjected to 40 psi of pressure (pressurized by using a bicycle pump) and also remained in the pressurized steel canister for 1 hour. The canister was then depressurized and the termites remained in the closed container for 24 hours. After such treatments, one to two termites from each group were randomly selected for confirmation of the effectiveness of the oxygenation and control treatment. Confirmation was achieved by examining 10µl of hindgut fluid, from a dissected termite gut, for presence of intact protozoa on a hemacytometer using a compound light microscope at 400X magnification (Chapter 1, fig. 4).

Antifungal Assay

In order to test the fungistatic ability of termite tissue, extracts of both faunated and defaunated termites were incubated with fungal conidia, then plated and colony forming units (CFUs) enumerated, following the methods outlined below. Immediately after the pressure only (faunated controls) and pressure plus oxygen (defaunated) treatments, termites were placed into glass dishes with a plaster of Paris substrate that was moistened with 1 ml of sterile water and maintained there for 24 hours. The plaster permitted termites to be kept under high moisture levels while eliminating feeding on
cellulose material (filter paper or wood). Hence, both defaunated and faunated treatments experienced the same degree of starvation and therefore we controlled for the effect of nutritional status on antifungal properties of the gut. Throughout the 24 hours on the moistened plaster, any residual glucanases from the hindgut symbionts (see Chapter 2) were likely flushed from the digestive system of the defaunated termites. Termites were then cold immobilized; each one’s gut was dissected by grasping the head with sterile tweezers and pulling out the gut through the anus with sterile fine-tipped tweezers. The entire gut was immediately placed into a Biomasher® (Cartagen) fitted with a filter (pore size 80-145 μm) and the corresponding head/gutted body of each individual was placed into a second Biomasher®. To each tube, 10 μl of a 200 mM sodium acetate buffer (NaAc; pH 5.0) was added to provide the correct pH for conidia germination. The contents of the tubes were then ground on ice with a pestle and centrifuged (4°C; 14,000 X g; 1 min.). After centrifugation, 20 μl of the supernatant was transferred into a 0.22 μm Ultrafree-MC® filter (Millipore) and centrifuged at (4°C; 12,000 X g; 4 min.) to filter out any bacterial contaminants. The filtrate was then mixed with 10 μl of a 0.2 mg/ml concentration of ampicillin (to control bacterial overgrowth) and 10 μl of a 1x10⁴ suspension of M. anisopliae conidia and incubated for 24 hours at 25°C while gently shaken at 50 RPM. Control samples (lacking termite tissues) were created with 10 μl sodium acetate buffer, 10 μl sterile distilled water, 10 μl of ampicillin, and 10 μl of M. anisopliae conidia suspension. Control samples were also incubated and shaken alongside the experimental groups. After 24 hours of incubation, 60 μl of sterile distilled water were added to each tube and 100 μl of the resulting solution were plated using
sterile glass beads onto PDA plates (100x15 mm) supplemented with 50 µg/ml of ampicillin and incubated at 25°C for 96 hours after which CFUs were counted.

**D-Glucono1,5-lactone (GDL) antifungal assay**

To test if potentially endogenous and symbiont-derived β-1,3 glucanases (βGlu) are responsible for the fungistatic or fungicidal function, termite extract combined with fungal conidia was incubated with and without D-Glucono-1,5-lactone (GDL), a specific inhibitor of termite βGlu (see chapter 2). Faunated and defaunated termite guts were dissected, as previously described, and placed in a Biomasher® (Cartagen) fitted with a filter (pore size 80-145 µm). Each tube contained either two guts or two head/gutted bodies of defaunated or faunated pressure-control termites. Ten µl of deionized water were added to each sample prior to centrifugation (4°C; 14,000 X g; 1 min.). Twenty µl of the centrifuged extract were transferred to a 0.22 µm Ultrafree-MC® filter (Millipore) and 20 µl of 200 mM NaAc was added. The tubes were again centrifuged (4°C; 12,000 X g; 14 min.), in order to filter out bacteria. The final extract was then divided into two aliquots and either used for the NaAc assay or the GDL assay. For the NaAc assay 10 µl of a 0.2 mg/ml ampicillin solution were added followed by 10 µl of a 1x10⁴ fungal conidia suspension. For the GDL assay the following reagents were added: 5 µl of a 0.4 mg/ml ampicillin solution, 5 µl of a 20 mM GDL solution, and 10 µl of a 1x10⁴ fungal conidia suspension. Control tubes were made for both NaAC and GDL following the above recipes, but in place of termite tissue they contained 10 µl of sterile water and 10 µl of 200 mM NaAc. All tubes were incubated for 24 hours at 25°C on a shaking platform (50 RPM). After 24-hours of incubation, 70 µl of sterile distilled water were
added to each tube and 100µl of the resulting solution were plated using sterile glass beads onto PDA plates (100x15 mm) supplemented with 50 µg/ml of ampicillin and incubated at 25°C for 96 hours after which CFUs were counted.

Statistics

To determine if colony of origin was a significant factor influencing fungistatic or fungicidal activity, CFU medians for each treatment were compared across colonies (Kruskal-Wallis; SPSS 17.0). To establish the effect of the various termite extracts on conidia viability (as measured in CFUs), the median CFUs were compared between treatments (Mann-Whitney U; SPSS 17.0). The Bonferroni correction was applied to all multiple pairwise comparisons to determine significance (p≤0.05/n, where n= the number of pairwise comparisons).
Results

Termite extracts, whether prepared from dissected guts or from head/gutted bodies, reduced conidia viability relative to that of control conidia. Consistent with our prediction, the fungistatic effect of guts was significantly stronger than that of the head/gutted bodies. This same pattern was observed across all six different termite stock colonies. Contrary to our prediction, the removal of symbionts (defaunation) still tended to reduce the median conidia viability across colonies. Incubation with GDL also had varying results across colonies with no evident trends. The effect of each of these variables is discussed, in turn, below.

Antifungal Assay

Effect of faunated termite extracts on conidia viability

Incubation of fungal conidia with gut and head/gutted body extracts from pressure-treated termites (faunated) in the majority of cases resulted in a reduction of fungal viability relative to control conidia (fig. 1). While the head/gutted body extract reduced the median number of CFUs in all colonies except colony D, the reduction in CFUs between head/gutted bodies and the corresponding controls was statistically significant only in colonies B and C (fig.1; Mann-Whitney U (MWU)=6.0, p=0.011; MWU=21.0, p=0.003, respectively; SPSS 17.0). In all six termite colonies examined, gut extracts significantly reduced fungal viability relative to the control treatment which lacked termite extracts (fig. 1; MWU, p<0.05; SPSS 17.0) Furthermore, in most colonies the fungistatic effects of the faunated gut extracts were significantly greater than that of the head/gutted bodies (fig 1).
Effect of defaunated termite extracts on CFUs

To test if the gut symbionts play a role in the fungistatic nature of termite guts, the extracts of defaunated termites were also incubated with fungal conidia. If symbionts produce antifungal metabolite(s), conidia incubated with extracts from defaunated termites should have increased viability relative to conidia incubated with faunated extracts. Both gut and head/gutted body extracts of defaunated termites reduced conidia viability relative to controls (fig. 2). However, contrary to our expectation, guts remained highly fungistatic even after defaunation. Guts of defaunated termites significantly inhibited conidia viability relative to controls, in all colonies tested (MWU, p<0.05; SPSS 17.0). While head/gutted bodies tended to reduce viability relative to controls, it was only a significant reduction in colony C (MWU=21.0, p=0.003; SPSS 17.0). A comparison of the fungistatic nature of defaunated vs. faunated guts did not show a consistent pattern across colonies (fig. 3). The only colony that showed a significant difference in antifungal activity between the faunated and defaunated guts was colony C (MWU=36.0, p=0.037; SPSS 17.0) in which the extracts of faunated guts were more fungistatic.

D-Glucono1,5-lactone (GDL) antifungal assay

To test if symbiont-produced βGlu’s play a role in fungal inhibition within the gut, termite extracts were incubated with a glucanase inhibitor, GDL, or with a control NaAc solution. There were no significant differences in conidia viability between any faunated extracts incubated with GDL and extracts incubated with the NaAc control solution (fig. 4). This was similarly true for defaunated extracts, with the exception of
two colonies (fig. 5): in colony F, the GDL incubated gut relative to the NaAc incubated gut significantly increased conidia viability (MWU, U=31.5, \( p=0.034 \); SPSS 17.0). However, in colony H the NaAc treatment relative to the GDL treatment exhibited a significant increase in conidia viability in the head/gutted body (MWU, \( U=0.000 \), \( p=0.05 \); SPSS 17.0). There were no significant differences across colonies in the CFUs of the control (without termite tissue extract) NaAc incubated conidia compared to the control GDL incubated conidia, indicating that the GDL alone had no negative effects on conidia viability. Overall, there were no consistent patterns observed in the various treatments of this assay.
Discussion

Our results demonstrate that relative to controls, viability of fungal conidia was significantly and negatively affected following incubation with extracts of termite tissue (whether the head/gutted body or gut extracts). Interestingly, the gut extracts had significantly higher inhibitory effects on conidia than body extracts. This was a consistent pattern across colonies (fig. 1) and points to the possibility that compounds in the gut, synthesized by either the termites themselves (endogenous compounds) and/or by the termite gut microbial communities (symbiont-derived compounds) are important in the deactivation of potential pathogens.

In order to test if this fungistatic nature of the gut was related to symbiont-produced metabolites, the strictly anaerobic portion of the hindgut community was removed via hyperbaric oxygen (Chapter 1, fig. 4). Because oxygen kills all the protists within the hindgut of lower termites as well as the strictly anaerobic bacteria (Cleveland, 1925a; Slaytor, 2000), we hypothesized that without the presence of these symbionts (and their secreted compounds), gut extracts should have been less effective in reducing conidia viability. However, with the exception of colony C (fig. 3), the fungistatic effects were still maintained when conidia were incubated with extracts of defaunated termites. In other words, regardless of the treatment (guts with intact microbial consortia or without normal microbial communities), gut extracts exhibited higher fungistatic activity than head/gutted body extracts. Therefore, contrary to our predictions, defaunated guts were not significantly less fungistatic then faunated guts, with the exception of colony C (fig. 3), indicating that in in vitro experiments, the anaerobic microbial community of Z. angusticollis may not be the sole source of the potent antifungal activity of the gut.
Additional *in vitro* experiments were performed to test if gut βGlu’s play a role in the fungal inhibition observed in termites. By adding D-Glucono1,5-lactone (GDL), a glucanase inhibitor, we expected to block the active site of such glucanases and thus, re-establish (rescue) conidia viability. These results were also questionable since no consistent effect on conidia viability was observed.

Although these later two *in vitro* data sets were inconclusive about the role that symbiont-produced compounds (i.e. βGlu’s) play in the gut’s antifungal properties, our results do not rule out the possibility of symbiont-mediated pathogen defense in *Z. angusticollis* (see Chapter 1). Below, we put forth several alternative explanations for why there seems to be a discrepancy between our predicted *in vitro* and our observed *in vivo* (Chapter 1) results.

*Symbionts as predators of pathogenic microorganisms*

The observed *in vivo* benefits of faunated termites relative to defaunated nestmates following fungal infection (Chapter 1) may be the result of protists engaging in predation of pathogenic microorganisms (Veivers et al., 1982). This is not unlikely as termite protists are known predators of bacteria (Inoue et al., 2000). Once conidia are ingested, termites would be capable of dealing with *per os* (by mouth) infections if their natural microbial consortia were to feed on the ingested material. But this benefit would obviously be lost during *in vitro* experiments as gut (and their accompanying microbial consortia) tissues were ground and filtered to produce the extract. Thus, the *in vitro* experimental protocols precluded any predatory opportunities by the gut microbiota.
**Bacterial overpopulation and compound compensation**

Several termite species have Actinomycetes in their gut (Bignell, 2000; Brauman, 2000; Brune 2006). Upon defaunation via oxygen, facultative anaerobes are poised to take advantage of the oxygen influx and can overpopulate the termite gut (Veivers et al., 1982). Given that facultative anaerobic Actinomycetes are thought to produce the fungistatic alkaloid, norharmane, in other termite species (Siderhurst et al., 2005), an increase in Actinomycetes densities following oxygenation could result in an increased concentration of norharmane in the gut. This could, in turn, cause a decrease in fungal viability. In other words, the loss of some symbiont-produced metabolites (i.e. glucanases) from the anaerobic protist community after oxygenation could be compensated by an increase of different metabolites produced by the overpopulation of the aerotolerant bacterial fauna.

*Insufficient GDL concentrations to block all of the multiple gut β-1,3 glucanases*

While it is possible that intact βGlu of *Z. angusticollis* guts do not play a role in fungal inhibition, a third potential explanation for the lack of congruency between our *in vitro* and *in vivo* experiments involves the ratio of added GDL to the number of active βGlu sites available for blocking. Because faunated guts of *Z. angusticollis* contain several βGlu’s (see Chapter 2), the GDL concentration used in our experiments may have not been high enough to block all the βGlu active sites, resulting in the inconsistent data observed amongst colonies. The guts and head/gutted bodies of defaunated termites, on
the other hand, typically have only two active βGlu (Chapter 2). The addition of GDL to these later extracts may have been enough to block the two remaining βGlu, resulting in the slight (but insignificant) increase in the CFUs seen in defaunated extracts (fig. 5). Based on our gels (Chapter 2, fig. 6), we are certain that GDL is indeed blocking the active sites of all the different glucanases. Yet, for the in vitro experiment we reduced the GDL concentration (from 100 mM to 20 mM), as previous in vitro experiments showed that GDL at high concentrations inhibited conidia germination. Previous work by Bulmer et al. (2009) used GDL to supplement the diet of the higher termite Nasutitermes corniger, which has only two known endogenous βGlu’s in the gut (as opposed to the dampwood termite, which has several symbiont-secreted βGlu’s in the gut; Chapter 2). This diet significantly increased pathogen related mortality. In our in vitro assay, blockage of active βGlu’s by GDL did not rescue conidia viability after incubation with gut extracts of faunated or defaunated Z. angusticollis. We conclude that, the GDL was not blocking all βGlu’s equally, thus resulting in the variability of the data. Further research will be required to fully understand the role of βGlu in Z. angusticollis pathogen defense.

While we have yet to fully pinpoint the role of βGlu in Z. angusticollis, it is clear that termite body tissues and in particular termite guts have strong fungistatic effects. Termites have several known secretions, both on and within their bodies that are used in defense against pathogens. For instance, defensive oral secretions of the soldiers of some fungus-growing termites are utilized by workers to suppress the growth of pathogenic fungi (Batra & Batra, 1966). Similarly, the frontal gland secretions of Nasutitermes spp. have a fungistatic effect on M. anisopliae (Rosengaus et al., 2000). Lower termites also
have chemical defenses such as secretions from the sternal gland, which have an inhibitory effect on fungi (Rosengaus et al., 2004). In addition to these secretions, other cuticular materials such as fatty acids, lipids and waxes may be important fungistatic agents, as they are in other insects (Koidsumi, 1957; Sannasi & Sundara Rajulu, 1967; Smith & Grula, 1982; St Leger, 1991). These various cuticular substances and secretions could explain the fungistatic nature observed in the head/gutted termite body.

Within the gut of Z. angusticollis, we have found strong antifungal activity, a result similar to what has been reported in other termite species (Kramm & West, 1982; Boucias et al., 1996; Siderhurst et al., 2005; Yanagawa & Shimizu, 2007; Chouvenc et al., 2009). While the production of norharmane by gut bacteria (Siderhurst et al., 2005) may partially explain the antifungal activity of the gut, it does not explain all the observed antifungal activity since norharmane’s effect on mycelial growth was less than 12% (Chouvenc et al., 2008, 2009). Physical attributes of the gut may also play a role in controlling fungal growth. For example, in living termites, the gut is partially anoxic (Brune et al., 1995) and an oxygen deficit can be inhibitory to fungal conidia (Cochrane et al., 1963). However, this does not explain the anti-fungal effect in termite cadavers two days post-mortem, as the microbe community responsible for maintaining the anaerobic gut condition is presumably dead (Chouvenc et al., 2009). While the pH of the gut could also influence conidia viability, the pH of Reticulitermes flavipes, (pH=6.0; Brune et al., 1995) should not have an influence on the germination and growth of M. anisopliae, as it is within its germination range (Dillon & Charnley, 1986). While it seems that the physical parameters of the gut may contribute to the fungistatic nature of the gut, their potential role appears limited.
Not only is the gut fungistatic, but the feces, which are used to line the nest walls and galleries of termite nests, are also fungistatic (Rosengaus et al., 1998b). The feces of *Z. angusticollis* contain active symbiont and putatively endogenous βGlu (see Chapter 2). Additionally, βGlu have been found in the nest materials of both *Z. angusticollis* (Bulmer & Rosengaus, unpublished) and *N. corniger* (Bulmer et al., 2009). It appears that termites are not only protecting themselves from fungal invasion, but are capable of excreting and incorporating those antimicrobial compounds into their nests. Although this research is the first to focus on the role that endogenous and symbiont-derived βGlu’s of *Z. angusticollis* play in termite pathogen defense, further research is required to pinpoint exactly the source of gut fungistasis. We believe the results of Chapters 1 and 2 provide strong circumstantial evidence that hindgut symbionts and their metabolites are important in controlling fungal development in the gut. Unfortunately, parts of our results of Chapter 3 are inconclusive. Future studies could include: determining the appropriate GDL concentration (between 20-100mM) required to block all glucanases in termite extracts without having negative effects on conidia viability, more selective methods of defaunation that narrow down which symbionts produce the βGlu’s, and incubation of conidia with *Z. angusticollis* isolated βGlu’s.
Figure 1: Relative percent colony forming units (CFU)* of conidia mixed with extracts of faunated termites. Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box. Bars with the same letter are not significantly different (P > 0.017) in pairwise comparisons within a colony (by MWU test adjusted with Bonferroni correction, SPSS)

*CFUs as percent of the median of the control treatment
**Figure 2:** Relative percent colony forming units (CFU)* of conidia mixed with extracts of defaunated termites. Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box. Bars with the same letter are not significantly different (P > 0.017) in pairwise comparisons within a colony (by MWU test adjusted with Bonferroni correction, SPSS)

*CFUs as percent of the median of the control treatment
Figure 3: Relative percent colony forming units (CFU)* of conidia mixed with gut extracts of faunated and defaunated termites. There were no significant differences in CFUs between guts of defaunated and faunated termites within each colony, with the exception of colony C (noted with an asterisk; MWU=36.0, p=0.037; SPSS 17.0). Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper edge of the box.

*CFUs as percent of the median of the control treatment
Figure 4: Relative percent colony forming units (CFU)* of conidia mixed with extracts of faunated termites and either sodium acetate (NaAc) or D-Glucono-1,5 lactone (GDL). Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper and lower edges of the box. There were no significant differences in pairwise comparisons within a colony (by MWU test adjusted with Bonferroni correction P<0.017, SPSS).

*CFUs as percent of the median of the control treatment
Figure 5: Relative percent colony forming units (CFU)* of conidia mixed with extracts of defaunated termites and either sodium acetate (NaAc) or D-Glucono-1,5 lactone (GDL). Each boxplot shows the median value and interquartile range. The outliers, identified by small circles, included cases with values between 1.5 and 3 box lengths from the upper and lower edges of the box. Significant pairwise differences were found between defaunated gut extracts of colony F (MWU=31.5, p=0.034) and between gutted bodies of colony H (MWU=0.0, p=0.05) both noted with an asterisk.

*CFUs as percent of the median of the control treatment
Conclusion

Insects, as a group, are highly successful and much of this success depends on their ability to resist diseases (reviewed in Loker et al., 2004). Insects depend on innate immunity to recognize and eliminate pathogens (Janeway & Medzhitov, 2002; Kvell et al., 2007). Typically, insects are not considered to have an adaptive immune response with specificity for foreign antigens or immunological memory (Janeway & Medzhitov, 2002). An adaptive immune system is more costly to maintain than an innate immune system (Kvell et al., 2007) and evolutionary ecology assumes that energetic resources are finite and organisms must allocate their resources to a limited number of tasks including immune defense (Schmid-Hempel, 2005). One possible way to supplement the innate immune system without significantly increasing resource investment by the host could be through a mutualistic pathogen defense symbiosis (Loker et al., 2004). Understanding the role of Z. angusticollis hindgut symbionts in pathogen defense could provide novel insights to understanding the role of mutualistic symbiosis in the development of immunity in solitary and social organisms.

Not only does research into symbiont-mediated pathogen defense open up new ways of understanding insect immunity, it also provides a different perspective to the evolution of host-mutualist interactions and the evolution of sociality in termites (see overall Introduction). Understanding eusociality has posed a particular conundrum to evolutionary biologists as giving up one’s own direct fitness in favor of the colony’s success is in direct opposition to Darwin’s theory of Evolution (Thorne, 1997). Yet the fact that it exists indicates its success. In other eusocial insects, the role of haplodiploidy
in the evolution of eusociality is strongly emphasized (Hamilton, 1964; Hamilton, 1972). However, termites are diploid and therefore do not have the skewed degrees of relatedness that are seen in the Hymenoptera (Thorne, 1997). To paraphrase, the existence of haplodiploidy is not a necessary pre-condition for the evolution of insect eusociality. A second hypothesis proposed to explain the evolution of termite sociality includes the need to acquire cellulolytic symbionts via social interactions, such as proctodeal feeding and coprophagy. Transfaunation of gut microbes may have been the driving force in the development of sociality in termites and other organisms (Cleveland et al., 1934; Jackson & Hart 2009). If the need for symbiont exchange promoted group living, this increased proximity to conspecifics could also increase the risks of infection. However, if symbionts provided nutritional support as well as pathogen defense, then their presence (and their associated metabolites) could help mitigate disease risks. Therefore, the influence of mutualistic symbionts on the evolution of sociality could be highly adaptive if nestmates benefited from improved abilities to breakdown cellulose while increasing their resistance against pathogenic microorganisms.

Our experiments provided strong evidence for symbiont-mediated pathogen resistance in the dampwood termite, Z. angusticollis. *In vivo* experiments illustrated that termites defaunated with oxygen are significantly more susceptible to infection by *M. anisopliae*. In Chapter 2, we identified β-1,3 glucanases (βGlu’s) produced by Z. angusticollis hindgut symbionts. βGlu’s are capable of binding to and breaking down β-1,3 glucans, which are common components of bacterial and fungal cell walls (Pitson et al., 1993; Brown and Gordon, 2005). While conclusive evidence as to their role in Z. angusticollis has yet to be obtained, βGlu’s are known to function in disease resistance in
the higher termite *N. corniger* (Bulmer et al., 2009). As the symbiont-produced βGlu’s are localized in the hindgut and *in vivo* experiments indicated the importance of hindgut symbionts in termite disease resistance, we designed *in vitro* experiments to test the influence of symbionts and symbiont-produced βGlu’s on *M. anisopliae* viability (Chapter 3). Our results demonstrated that *Z. angusticollis* tissue is fungistatic and that guts in particular are highly fungistatic. Removal of the strictly anaerobic hindgut symbionts does not change the fungistatic nature of the gut. Additional attempts at blocking the βGlu’s also had no significant effect on fungal viability. Our collective results (from the three chapters) point to the possibility that the fungistatic nature of the gut reported in several termite studies (Kramm & West, 1982; Boucias et al., 1996; Rosengaus et al., 1998a; Yanagawa & Shimizu, 2005) may be a combination of multi-layer interactions including (but not limited to) the termite’s own immune defense, the biochemical environment of the gut, predation of pathogens by protists, and symbiont-produced enzymes. Understanding symbiont-mediated pathogen resistance appears to be critical to developing more accurate evolutionary scenarios and providing deeper insights into the interactions between the evolution of insect immune defenses and eusociality.
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