"Antifungal defenses in subterranean termites and *Cryptocercus* woodroaches"

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Diandra Denier entitled Antifungal defenses in subterranean termites and *Cryptocercus* woodroaches, has been approved by her committee as satisfactory completion of the requirement for the degree of Master of Science.

Chair, Thesis Committee — Date 1/11/13

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Special thanks to my supportive boyfriend, Kenneth Kirwan, for always being willing to lend an ear and listen to my research. Also thanks to my family for being behind me every step of the way.

Abstract

Diandra Denier

The secreted β-1,3-glucanase activity of Gram-negative bacteria binding proteins (GNBPs) provides woodroaches with important prophylactic protection from fungal pathogens such as *Metarhizium anisopliae*. Cuticular washes have antifungal activity against *M. anisopliae* conidia that was suppressed by an inhibitor (GDL) of termite GNBP β-1,3- glucanase activity. *Cryptocercus punctulatus* nymphs that were treated with GDL and subsequently exposed to *M. anisopliae* conidia show significantly greater mortality than the untreated nymphs exposed to conidia. The β-1,3- glucanase activity of GNBPs therefore appears to be critical for protecting *Cryptocercus* woodroaches from fungal pathogens.

Analysis of local and foreign *Metarhizium* strains indicates that *Metarhizium* has the potential to influence the evolution of the termite immune system. To investigate *Metarhizium* strain variety and virulence, six strains were isolated and identified from nearby *Reticulitermes flavipes* collection sites. Colonies varied significantly in their susceptibility to the six isolates of *Metarhizium*, which were collected across a rough transect of approximately 1 km. These fungal isolates represented three separate species, *M. brunneum*, *M. robertsii* and *M. guizhouense*. There was a significant correlation between the genetic distance between isolates and their difference in virulence in three of

four termite colonies. This variety of *Metarhizium* over small spatial scales suggests that adaptive evolution in the termite immune system may arise as a result of a virulent *Metarhizium* strain periodically creating epizootics that are countered by the evolution of resistance in the host.

Messenger RNA sequences of Gram Negative Bacteria-binding Protein 1 (*GNBP1*) were identified and analyzed in two species of subterranean termites. Using population genetic methods, comparisons were made between this gene and two additional antifungal genes in the subterranean termites and two species of woodroaches, *Cryptocercus punctulatus* and *Cryptocercus wrighti*. An analysis of nucleotide intraspecific polymorphism indicated that these genes frequently face selective sweeps, possibly as a result of a virulent fungal strain spreading through populations and selecting for resistant specific alleles that afford the greatest resistance to infection.

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Chapter 1

A common antifungal defense strategy in *Cryptocercus* woodroaches and termites

Abstract

In termites, the secreted β-1,3-glucanase activity of Gram-negative bacteria binding proteins (GNBPs) provides important prophylactic protection from fungal pathogens such as *Metarhizium anisopliae*, which can evade the immune system after entering the insect. Termites evolved from a cockroach-like ancestor that is believed to resemble *Cryptocercus* woodroaches. Here, β-1,3-glucanase activity is identified on the cuticular surface of the woodroach *Cryptocercus punctulatus* that originates from the salivary gland and is likely spread by allogrooming. Cuticular washes have antifungal activity against *M. anisopliae* conidia that is suppressed by an inhibitor (GDL) of termite GNBP β-1,3- glucanase activity. *C. punctulatus* nymphs that are treated with GDL and subsequently exposed to *M. anisopliae* conidia show significantly greater mortality than the untreated nymphs exposed to conidia.

Introduction

Despite living in crowded conditions and microbe-rich environments that promote the rapid spread of disease, social insects such as ants and termites are among the most abundant of terrestrial animals (Wilson, 1999). Two antifungal proteins, termicin and Gram-negative bacteria binding protein 2 (GNBP2), have been identified in termite salivary glands and the β-1,3-glucanase activity of GNBP has been detected on the insect cuticle as well as in nest-construction materials (Lamberty *et al.*, 2001; Bulmer *et al.*, 2010). These GNBPs are spread across the cuticle by allogrooming to provide protection against pathogenic fungi such as the ubiquitous soil pathogen *M. anisopliae* that can directly penetrate through the cuticle. The fungal cell wall is easily disrupted by GNBP2 which allows termicins to access and fatally interfere with the integrity of the cell membrane or to destroy the cell by some other mechanism (Bulmer *et al.*, 2009; Rosengaus *et al.*, 2011).

Termites evolved in the Late Jurassic from a cockroach-like ancestor that is likely to have shared subsocial characteristics with woodroaches belonging to *Cryptocercus*, which represent the closest living relatives to termites (Lo *et al.*, 2000; Inward *et al.*, 2007; Engel *et al.*, 2009; Ohkuma *et al.*, 2009). Like termites, *Cryptocercus* may be especially vulnerable to the spread of pathogens, because they live in crowded conditions in a microbe-rich environment. *Cryptocercus* adults and nymphs live in decaying wood in family groups that are frequently composed of 30 or more individuals (Nalepa, 1988). These insects have a nitrogen poor diet and avoid heavy nitrogenous investment in the development of the external cuticle until late developmental stages (Bell *et al.*, 2007; Nalepa, 2011). Prolonged development of four or more years (Bell *et al.*, 2007) with a thin cuticle is likely to increase a nymph's vulnerability to *Metarhizium* conidia, which can rapidly grow through thin cuticle.

Secreted antifungal GNBPs that are spread by allogrooming may be critical for protecting *Cryptocercus* species from fungal pathogens given that *Cryptocercus* and termites share traits such as group-living, allogrooming and thin cuticles in early developmental stages. Inhibition of GNBP2 β-1,3-glucanase activity with glucono delta

lactone (GDL) significantly increases the susceptibility of *Nasutitermes corniger* workers to a laboratory strain of *M. anisopliae* (Bulmer *et al.*, 2009) and *Reticulitermes flavipes* workers to naturally encountered strains of *M. anisopliae* (Hamilton *et al.*, 2011). In this study, we identified and characterized GNBPs and their predicted antifungal activity in the woodroach *Cryptocercus punctulatus*, which is likely to share many characteristics with the subsocial ancestor of *Cryptocercus* and termites.

Materials & Methods

Woodroach collection

Cryptocercus punctulatus were collected from decayed wood approximately 3 km west of Huntly, Virginia, in November 2010 and August 2011. *Cryptocercus* were maintained in plastic containers with pieces of wood at 25 °C in constantly dark environment and kept moist by spraying with water once a week. RNA was extracted from nymphs by gently crushing them in RNA later and stored at -20 °C until purified with a Quick Prep mRNA kit (Amersham Biosciences). In addition, insects were stored for DNA preparation at –20 °C in 100 % ethanol. DNA was prepared with a Qiagen DNA purification kit.

Cockroaches were identified using morphological characteristics, geographical location and comparisons of mitochondrial 16S rRNA to known sequences in GenBank (Ye *et al.*, 2004).

Metarhizium anisopliae isolation

Local fungal strains of *M. anisopliae* were isolated from soil samples taken from nearby each termite and woodroach collection site. Soil samples were free of termites or woodroaches. The baiting technique involved placing five *Tenebrio molitor* larvae, which are highly susceptibility to infection by the fungus, in to 50 g of soil and monitoring for mealworm deaths. Bodies were then surface sterilized with 70% ethanol and set aside in petri dishes with moist filter paper for a 5-7 days before being checked for confirmation of *M.anisopliae* infection. Conidia were collected from individuals that died of infection with a sterile inoculation loop, streaked on potato dextrose agar and grown at 25 ºC to obtain single clonal strains that were further cultivated to produce conidia. The identity of *M. anisopliae* was confirmed by comparing the ITS region, amplified with the universal primers PN3 and PN34, with GenBank sequences (Viaud *et al.*, 2000).

β-1,3-Glucanase activity of cuticular washes

Three *C. punctulatus* nymphs, measuring approximately 1.5 cm in length, were chilled and rinsed with 50 µL of Tween 80 kept at 4ºC. Nymphs were placed in 1.5mL polypropylene tubes with Tween 80 consecutively and gently agitated for 10 seconds. The cuticular washes were filter sterilized with 0.22 µm Amicon microcentrifuge tubes and assayed by gel electrophoresis using a 12 % polyacrylamide gel with 0.13 % carboxymethyl curdlan-Remazol Brilliant Blue (Loewe Biochemica). Gel slices were incubated in 100 mM sodium acetate, pH 5, and with or without 100mM GDL for 24 hours. Clearing zones representing enzymatic activity were photographed.

In vitro antifungal assay of cuticular washes

40 µL of filter sterilized cuticular washes or 0.1% Tween 80 (control) were incubated with approximately 100 *M. anisopliae* conidia with or without 100 mM GDL for 24 hours. Total washes with conidia (4 replicates with or without GDL) and controls (8 replicates) were plated on potato dextrose agar with a sterilized glass-rod spreader and incubated for 3–4 days at room temperature. The average number of germinated conidia or colony-forming units (CFUs) was compared among treatments using an ANOVA followed by a Tukey's HSD test.

In vivo antifungal assay

Three cohorts of young lightly sclerotized nymphs from separate families (n = 16 per cohort), measuring approximately 1.5 centimeters along the dorsal surface from head to the end of the abdomen, were divided into two replicates of 4 individuals for both control and GDL treatments prior to fungal challenge (total n = 48). The replicates of four nymphs for each cohort were placed in dishes (60 mm) with sterile filter paper (Whatman 3) moistened with 600 µL of 100 mM sodium acetate (pH 5) (NaOAc treatment) or 300 mM GDL/100 mM sodium acetate (pH 5) (GDL treatment) for 24 h. Nymphs from the NaOAc and GDL treatments were then divided into control or challenge groups. Control groups (n = 4 for each cohort) were placed on filter paper (Whatman 5) moistened with

300 µL of 0.1 % Tween 80 and challenge groups (n = 4 for each cohort) were placed on filter paper moistened with 300 µL of 10^7 conidia mL^{-1} for 24 h. The nymphs were challenged with a local strain of *M. anisopliae* (Rappahannock County, VA, sample). After 24 h, nymphs were placed on filter paper (Whatman 3) moistened with 600 µL of distilled water alone, and survival was monitored daily for 21 days. Dead nymphs were removed, surface-sterilized with 70 % ethanol, and placed on moist sterile filter paper to confirm the infection by *M. anisopliae*.

Results

β-1,3-Glucanase and antifungal activity

Brief 10 second washes of the cuticle of *C. punctulatus* nymphs with a surfactant (0.1 % Tween 80) show β-1,3-glucanase activity (a single clearing zone) in the CM-curdlan-RBB gel electrophoresis assay, and this activity is inhibited by GDL (Fig. 1a), a specific inhibitor of β-1,3-glucanase activity (Bulmer *et al.*, 2009). The cuticular washes also show significant antifungal activity against *M. anisopliae* conidia, which is inhibited by GDL (Fig. 2). This indicates that the β-1,3-glucanase activity is an important component of the antifungal activity. The cuticular wash clearing zone corresponds with the salivary gland clearing zone (Fig. 1b). The slight difference in the electrophoretic mobility of the cuticular wash and salivary gland β-1,3-glucanases is attributable to greater enzyme activity in the salivary gland sample. CM-curdlan-RBB impedes the migration of β-1,3-glucanases in the gels. The diffuse clearing zone above the salivary gland band indicates that the β-1,3-glucanase activity digested some of the curdlan during electrophoresis, which would have reduced curdlan's ability to impede migration of the β-1,3-glucanase. Other termite tissues including the alimentary tract have β-1,3-glucanase clearing zones that correspond with the salivary gland clearing zone. There appear to be additional unique β-1,3-glucanases in the alimentary tract (lane 2, Fig. 1b).

Figure 1. CM-curdlan-RBB polyacrylamide gels. a) C. punctulatus cuticular washes, b) C. punctulatus tissue extracts. From left lane to right: lane 1 extract without the salivary gland or alimentary tract, lane 2 alimentary tract, lane 3 salivary gland, lane 4 cuticular wash.

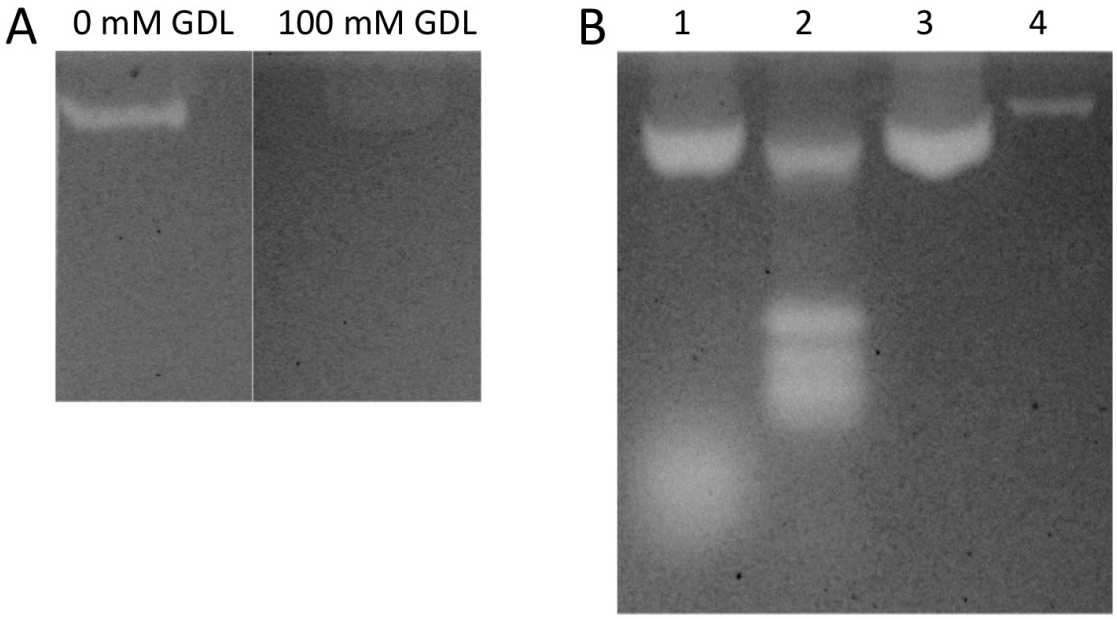
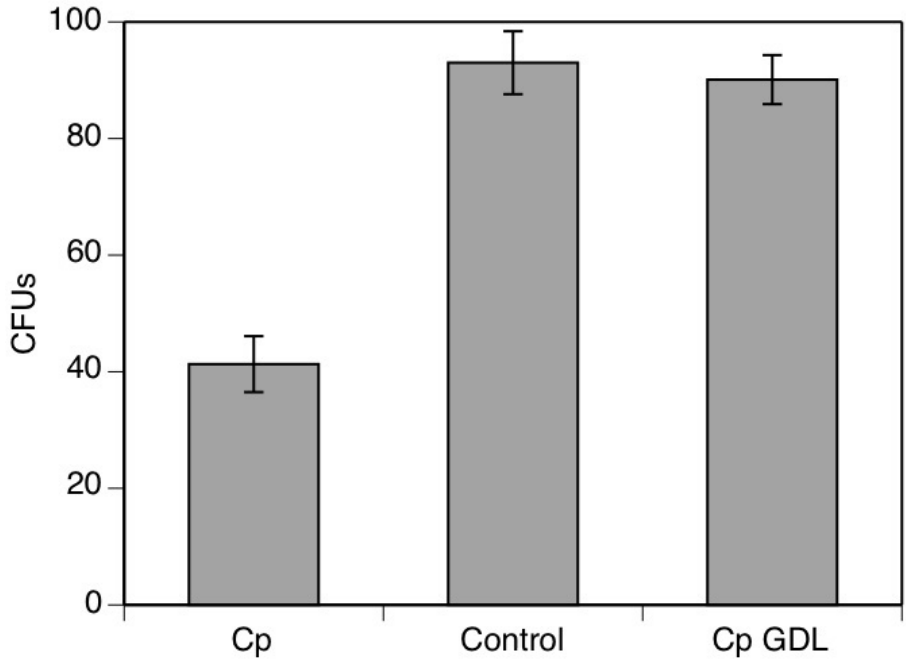


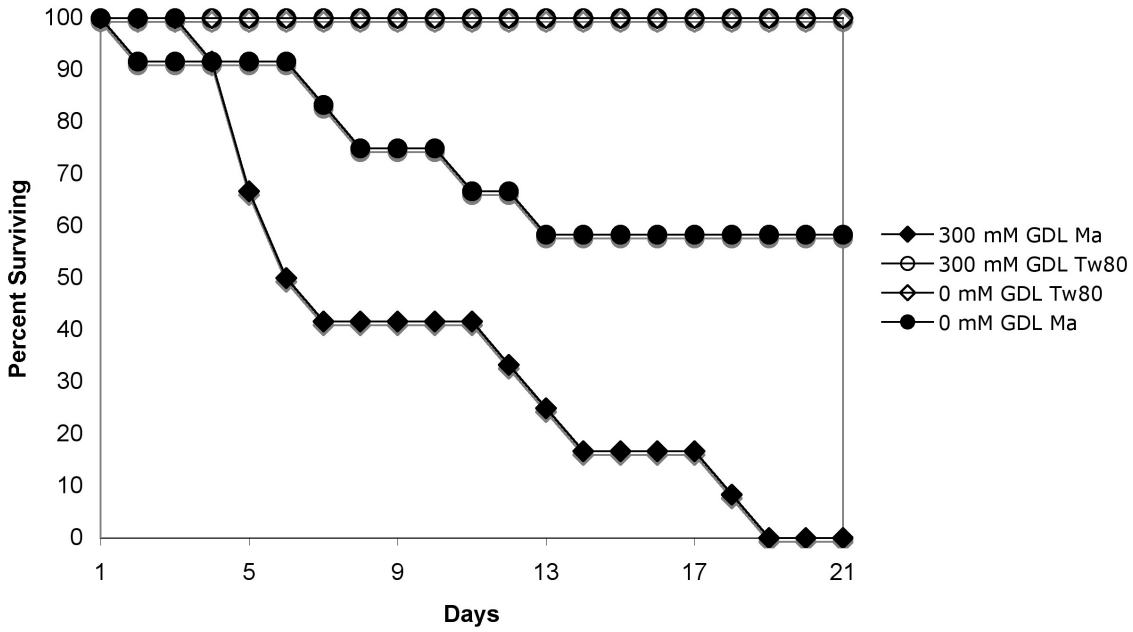
Figure 2. Colony-forming units of *M. anisopliae* conidia after treatment with *C. punctulatis* cuticular 0.1 % Tween 80 washes (Cp), 0.1 % Tween 80 alone (control) or washes and 100 mM GDL (Cp GDL) ($F_{2,13} = 30.2$, $p < 0.001$). The number of CFUs for Cp but not Cp GDL were significantly lower than CFUs in the control (Tukey's HSD, $p < 0.001$ and $p = 0.907$, respectively). GDL alone has been shown to have no effect on CFU counts with the specific strain and isolate used in this experiment (Hamilton *et al.*, 2011). The error bars represent standard deviations.



GDL β-1,3-glucanase inhibition and nymph survival

Treatment with GDL prior to a fungal challenge with *M. anisopliae* increased *C. punctulatus* nymphs' susceptibility to infection (Fig. 3). Nymphs treated with GDL prior to a fungal challenge exhibited a 4.4 times higher hazard ratio of death relative to NaOAc control challenged nymphs (Wald = 7.3, df = 1, p = 0.007). *M. anisopliae* infection was confirmed in all nymphs that were exposed to the fungal pathogen and died in the course of the experiment. Melanotic lesions (dark spots) were also observed in all nymphs that were exposed to *M. anisopliae* and are likely to represent points at which germinating conidia penetrated the cuticle. Treatment with GDL alone resulted in no mortality indicating that GDL had no adverse effect on survival. Cohort effect on survival was not significant (Wald = 2.0, df = 2, p = 0.37).

Figure 3. Woodroach survival after GDL and *M. anisopliae* exposure. Survival of young nymphs from three cohorts of *C. punctulatus* (n = 48) treated with 0 mM GDL or 300 mM GDL prior to exposure to *M. anisopliae* conidia (Ma). Controls were exposed to 0.1 % Tween 80 (Tw80).



Discussion

Cryptocercus species secrete antifungal GNBPs and spread them by auto- and allogrooming. In support of this hypothesis, washes of the cuticle of *C. punctulatus* nymphs with a surfactant (0.1 % Tween 80) have β-1,3-glucanase activity and this activity is inhibited by GDL (Fig. 1a), a specific inhibitor of the β-1,3-glucanase activity associated with GNBPs (Bulmer *et al.*, 2009). Cuticular washes show antifungal activity against *M. anisopliae* conidia and this activity is also inhibited by GDL (Fig. 2). This indicates that the antifungal activity of the washes depends on β-1,3-glucanase activity that appears to originate from the salivary gland (Fig. 1b), which is a known source of termite GNBPs with β-1,3-glucanase activity (Yuki *et al.*, 2008; Bulmer *et al.*, 2009). *In vivo* treatment with GDL significantly increases the susceptibility of nymphs to infection from a local strain of M. anisopliae (Fig. 3). The β-1,3-glucanase activity of salivary gland secretions that are spread by allogrooming, therefore, appears to be essential for protecting *Cryptocercus* nymphs from their fungal pathogens.

A β-1,3-glucanase clearing zone corresponding with the salivary gland is also seen with extract of the alimentary tract or extract in which the salivary gland and alimentary tract had been removed (Fig. 1b), which indicates that *C. punctulatus* tissues other than the salivary gland produce this β-1,3-glucanase. The β-1,3-glucanase activity of GNBP2 is found in the hemolymph of *Nasutitermes corniger* where this protein appears to function as a PRR (Bulmer *et al.*, 2009). It may also act as an effector that can limit the invasion of fungal pathogens into the hemocoel. Clearing zones, which are associated with the alimentary tract and not the cuticle or salivary gland, may represent hindgut symbiont β-1,3 glucanases. *Cryptocercus* and termite hindgut protist communities are derived from a common ancestral community (Ohkuma *et al.*, 2009). β-1,3-Glucanase clearing zones associated with the hindgut in termites that contain protists are absent in *N. corniger* that lacks protists, which suggests that the activity is attributable to protists or their endosymbionts (Bulmer *et al.*, 2009, compare Fig. 3c and S9). β-1,3-Glucanases in the alimentary tract are likely to be important for inactivating ingested

conidia that enter the tract after grooming or cannibalism of infected individuals (Bell *et al.*, 2007; Chouvenc *et al.*, 2010).

A thin cuticle in woodroach nymphs may have been important for the evolution of an external antifungal defense system. This externalized defense could have facilitated the evolutionary transition from a sub-social woodroach ancestor that lived in simple families to eusocial termites that live in crowded colonies. As a result of crowded conditions in colonies, termites are expected to be more vulnerable to infection by pathogens such as *Metarhizium* because termites frequently exchange food and symbionts and groom each other. This frequent contact can potentially spread fungal conidia to uninfected individuals, which would severely impact the health of the colony if the externalized antifungal defense system was not present or effective.

Chapter 2

Variation in subterranean termite susceptibility to indigenous *Metarhizium* species

Abstract

Molecular evolutionary studies have revealed that components of the termite innate immune system responsible for antifungal defense are adaptively evolving. *Metarhizium* species, which are important fungal pathogens of termites, reproduce asexually and are usually described as generalists that infect more than one host species. This fungus therefore appears to be an unlikely candidate for driving adaptive evolution in termite and woodroach antifungal proteins. However, an analysis of *Metarhizium* strains that subterranean termites encounter naturally indicates that *Metarhizium* has the potential to influence the evolution of the termite immune system. To investigate *Metarhizium* strain variety and virulence, we isolated and identified *Metarhizium* samples in the immediate vicinity of subterranean termite colonies belonging to *Reticulitermes flavipes*. Variation in virulence of each of strain was tested with an *in vivo* assay of termite survivorship after exposure to fungal conidia. Colonies varied significantly in their susceptibility to six isolates of *Metarhizium* collected across a rough transect of approximately 1 km. These fungal isolates represented three separate species, *M. brunneum*, *M. robertsii* and *M. guizhouense*. There was a significant correlation between the genetic distance between isolates and their difference in virulence in three of four termite colonies. This variety of *Metarhizium* over small spatial scales suggests that adaptive evolution in the termite immune system may arise as a result of a virulent *Metarhizium* strain periodically creating epizootics that are countered by the evolution of resistance in the host.

Introduction

Subterranean termites depend on the innate immune system for defense against microbial pathogens. Effectors of this system are externalized to prevent infection from fungi such as *Metarhizium anisopliae* that can evade the insect immune system after growing through the cuticle (Wang and St Leger, 2006). The externalized effectors include termicins and β-1,3-glucanases, which appear to work together to destroy fungal conidia or germinating mycelia, and appear to be particularly effective against *Metarhizium anisopliae* (Bulmer *et al.,* 2009; Hamilton and Bulmer, 2012; Hamilton *et al.,* 2011). Termite termicins and β-1,3-glucanases show unusually strong signatures of adaptive evolution (Bulmer and Crozier, 2004; Bulmer and Crozier, 2006; Bulmer *et al.,* 2010), which indicates that they face strong selective pressure from fungal pathogens.

Subterranean termites are inevitably exposed to *Metarhizium* strains that are ubiquitous in soils. Termite worker traffic through their extensive network of subterranean galleries exposes workers to conidia that can attach to the cuticle and this traffic may even help distribute conidia to new locations. *Metarhizium* species can potentially infect a wide range of arthropod hosts and are often considered to be a generalist pathogen that is not restricted to one host (Roberts and St Leger 2004). The signatures of adaptive evolution in termicins and β-1,3-glucanases, which include evidence of selective sweeps that drive positive selection (Bulmer *et al.,* 2010), are usually associated with an arms race between a specific host and pathogen over many generations. The evolution of resistance in the host is countered by the ability of the pathogen to evade or usurp the immune system. If both the pathogen and host sexually reproduce then the pathogen may often have the upper hand in this race as it typically has a shorter generation time than the host. The *Metarhizium* species that subterranean termites are likely to encounter reproduce asexually. Termicins and β-1,3-glucanases are highly effective against *Metarhizium* isolates collected adjacent to subterranean termite foraging workers (Hamilton and Bulmer, 2012; Hamilton *et al.,* 2011). However it is unclear whether an asexually reproducing generalist could be driving adaptive evolution in termicins and β-1,3-glucanases.

Studies of the types and variety of *Metarhizium* strains that subterranean termites encounter naturally over small spatial scales could provide insight into whether *Metarhizium* has the potential to influence the evolution of the termite immune system. Termites might be more susceptible to infection from *Metarhizium* strains that occur in close proximity to their colonies than more distant strains, which would reflect local adaptation of the fungal pathogen to its host. Termites might also be more susceptible to particular strains that have evolved specific mechanisms for infecting termites. To investigate *Metarhizium* strain variety and virulence, we isolated and identified *Metarhizium* samples in the immediate vicinity of subterranean termite colonies belonging to *Reticulitermes flavipes* over a small spatial scale, a rough transect of approximately 1 km. The potential variation in virulence of each of these strains against four colonies of *R. flavipes* was tested with an *in vivo* assay of termite survivorship after exposure to fungal conidia.

Materials & Methods

Fungal Isolation

M. anisopliae was isolated from soil samples by a baiting technique that takes advantage of *Tenebrio molitor* larval (mealworms) susceptibility to infection by *M. anisopliae* (Hughes *et al.,* 2004). Larvae were placed in small plastic containers together with soil samples and monitored daily. Dead larvae were removed, surface sterilized with 70% ethanol and kept covered in petri dishes on moist filter paper. Dead larvae infected with *M. anisopliae* develop a distinct green muscadine appearance. Conidia were harvested from the cadavers and clones from conidia grown up on potato dextrose agar plates supplemented with 50 μg/ml ampicillin.

Conidia solutions were made from the conidia grown on PDA/Amp plates with 0.1% Tween-80 (Tw80), a solution that prevents the conidia from clumping. A flame sterilized platinum wire loop was moistened with Tw80 and scraped over the conidia. The loop was then immersed and agitated in Tw80 to release the conidia from the loop. This was repeated with vortexing of the Tw80 conidia solution to achieve a stock solution with a high concentration of conidia. Dilutions were made from the stock and a hemocytometer was used to calculate conidia concentrations.

Molecular methodology

M. anisopliae was identified by 1) purification of fungal DNA from conidia and mycelia, 2) PCR amplification of an ITS region (partial sequence of 18S ribosomal RNA, the internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and partial sequence 28S ribosomal RNA gene) using PCR primers PN3 and PN34 (Viaud *et al.,* 2000), the flanking IGS region using PCR primers Ma-IGSspR and Ma-IGSspF (Pantou *et al.,* 2003), the 5' region of EF1- alpha using PCR primers EF1T and EF2T (Bischoff *et al.,* 2006), and 3) DNA sequencing of the PCR products.

Different strains and species were identified by comparing the sequence with the ITS, IGS and EF1-alpha sequence in established databases such as GenBank (NCBI). The sequences were aligned manually with the program Se-Al (Rambaut, 2002) and

maximum-likelihood trees were constructed with the program PAUP* (Swofford, 2002). The optimum substitution models were determined with the programs Modeltest (Posada and Crandall, 1998) and PAUP* (Swofford, 1998).

The correlation between genetic and physical distance was assessed with a Mantel test, which is a widely used method for assessing the relationships between two distance matrices (Bonnet and Van de Peer, 2002). The Mantel test was also used to test for a correlation between genetic distance and the difference in the hazard ratios of death for six strains of *Metarhizium* relative to Tw80 controls. This mantel test was repeated in four *R. flavipes* colonies. Tests were performed with the program *zt* using 10,000 randomizations (Bonnet and Van de Peer, 2002). Pairwise genetic distances between the strains were calculated with the maximum-likelihood distances used to construct the phylogeny from IGS and ITS DNA sequence. Hazard ratios for each *Metarhizium* isolate were calculated from a Cox regression analysis of termite survivorship by comparing the survivorship with *Metarhizium* treatment to the Tw80 controls.

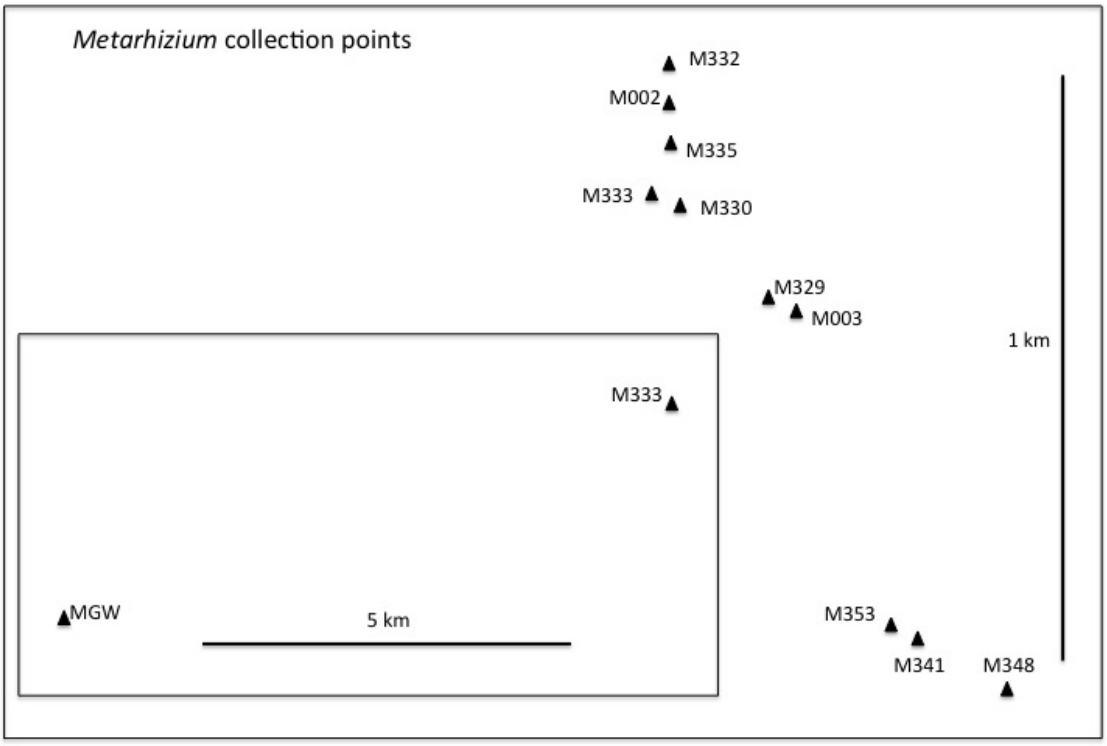
In vivo assay

Reticulitermes flavipes workers were collected from decayed wood in Towson, Maryland, USA, during July and August of 2008 and 2011. Collection points are likely to have been sufficiently separated (> 85 meters) to insure that they represented workers from different colonies (Vargo and Husseneder, 2009). Termites were maintained in plastic containers with pieces of wood at 25 °C in constant dark and kept moist by spraying with water once a week. Soil samples were taken from nearby (< 30 cm) foraging termite workers (designated as colonies) and were free of any foraging termites. *Metarhizium* strains were isolated from each soil sample with the *Tenebrio molitar* larvae baiting technique.

Termite colony resistance to both local (within 30 cm of termite collections) and distant fungal strains of *Metarhizium* was tested with approximately 180 worker termites per colony (n=4). Termites were acclimatized to petri dishes with filter paper saturated with dH_2O for two days prior to the start of the experiment. Termites were treated with a 10^7 conidia mL^{-1} solution, which caused moderate mortality in preliminary studies.

The termites were divided into replicates of 12 per experimental condition. Termites were placed in petri dishes on Whatman (#5) filter paper that had been moistened with 300uL of 10^7 conidia mL^{-1} solution or 300 µL of 0.1% Tw 80. Local strains were isolated from the soil taken nearby the specified termite colony, while distant strains were those that had been isolated from soil adjacent to the other termite colonies used in the experiment. This was repeated for each strain of fungus including M002, M353, M348, M003, M335 and MGW (Figure 1).

Figure 1. Map showing the genetic distances between the different *Metarhizium* isolates used in the study. All isolates were collected from the same continuous wooded area, except MGW, separated from the others by an urban area (Baltimore County, MD).



Workers from four colonies were tested against their local *Metarhizium* strain and the five other distant strains. The termites were allowed to walk around on the filter paper and ingest the conidia or Tw80 as they chewed the filter paper. After 24 hours, the filter paper was removed and replaced with filter paper moistened with 300 μL of dH_2O. All petri dishes were maintained in containers with moist Kim wipes to prevent the termites from desiccating. Termite survival was monitored for 21 days. Deceased termites were removed, surface sterilized with 70% ethanol and placed on moist filter paper in a petri dish for confirmation of *Metarhizium* infection. Termite survivorship and hazard ratios of death were analyzed with Cox regression.

Results

Phylogenies and Species Identification

The IGS and ITS sequences indicate that there is considerable genetic diversity in the *Metarhizium* isolates over short distances. The maximum-likelihood tree for IGS and ITS is congruent with the tree for EF1 (Figures 2 and 3). The BLAST analysis of these sequences indicated that the isolates correspond with three different species of *Metarhizium* (for example, Table 1), which are indicated with brackets in the phylogenies.

Figure 2. Maximum-likelihood tree with bootstrap values for the concatenated IGS and ITS sequence. The K81+G substitution model was used for constructing the tree. The *Metarhizium* isolates collected adjacent to colonies used in the termite survival analysis are highlighted in blue. The scale bar corresponds with 10 nucleotide substitutions. The combined aligned sequence is 828 nucleotides.

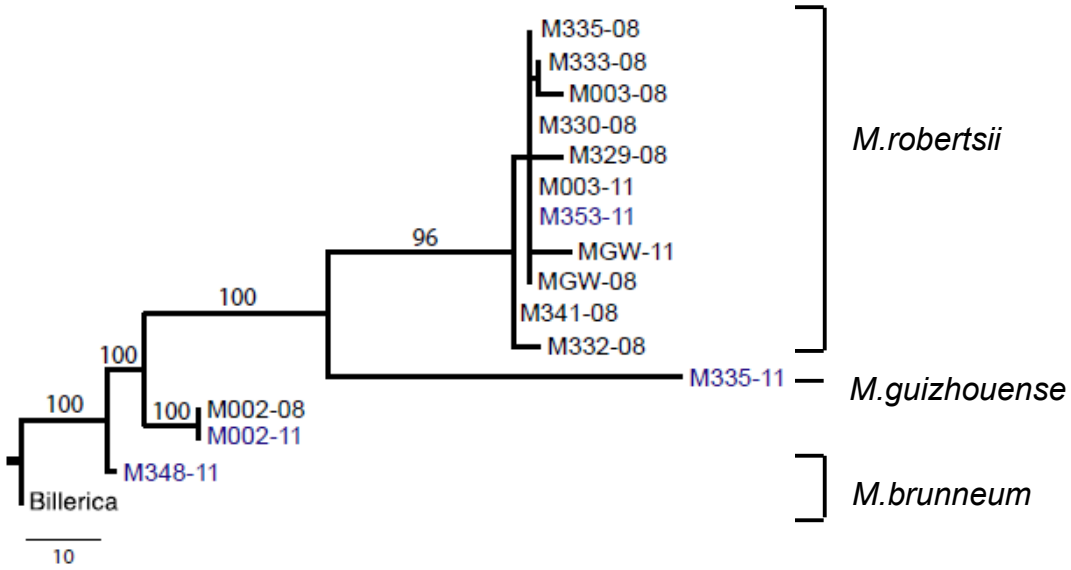


Figure 3. Maximum-likelihood tree with bootstrap values for the 5' region of EF1. The K81 substitution model was used for constructing the tree. The scale bar corresponds with 7.5 nucleotide substitutions. The aligned sequence is 711 nucleotides.

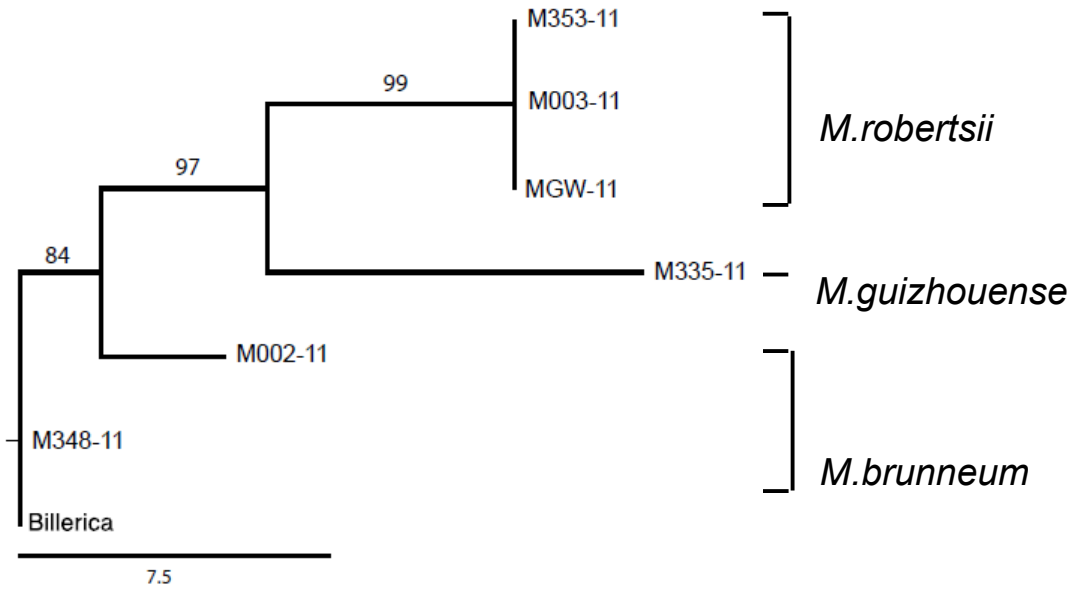


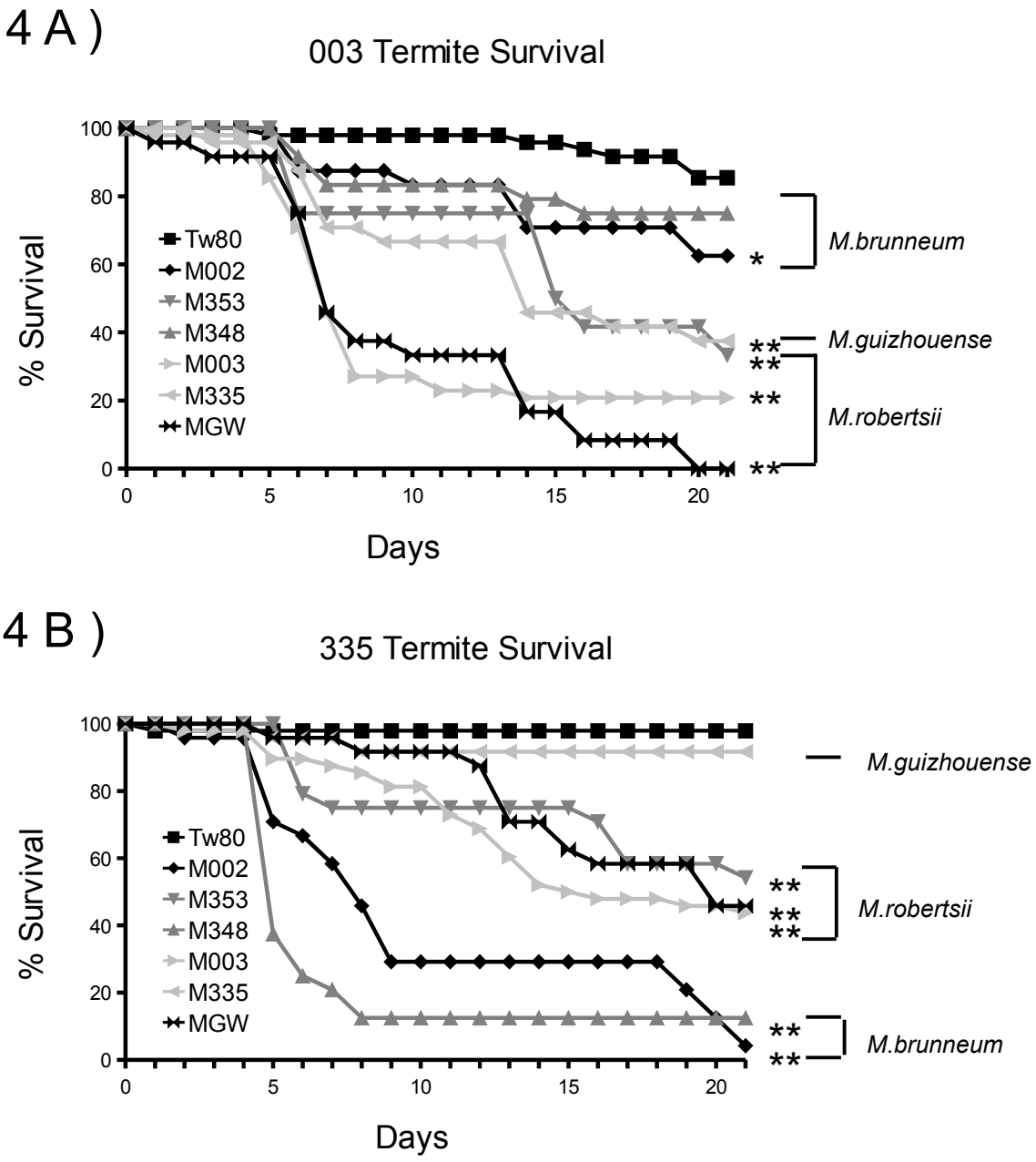
Table 1. BLAST identities of EF1 sequence

strain	BLAST match	identity
M335-11	*M. guizhouense*	710//710
M002-11	*M. brunneum*	711/711
M348-11	*M. brunneum*	707/707
M353-11	*M. robertsii*	708/708
M003-11	*M. robertsii*	708/708
MGW-11	*M. robertsii*	708/708
Billerica	*M. brunneum*	707/707

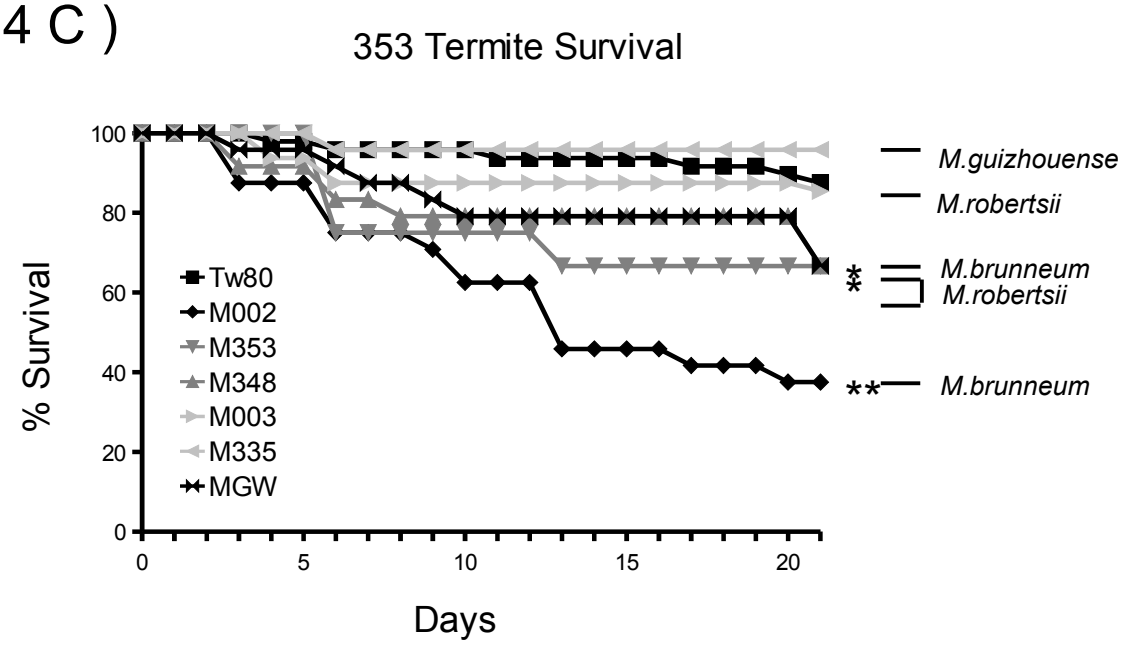
In vivo analysis

The survivorship analysis indicated that the four colonies varied in their vulnerability to infection by the *Metarhizium* isolates. There was an apparent hierarchy in colony vulnerability to lethal infections with colony GF2 having the greatest resistance to infection followed by 353, 335 and 003 (Figure 4).

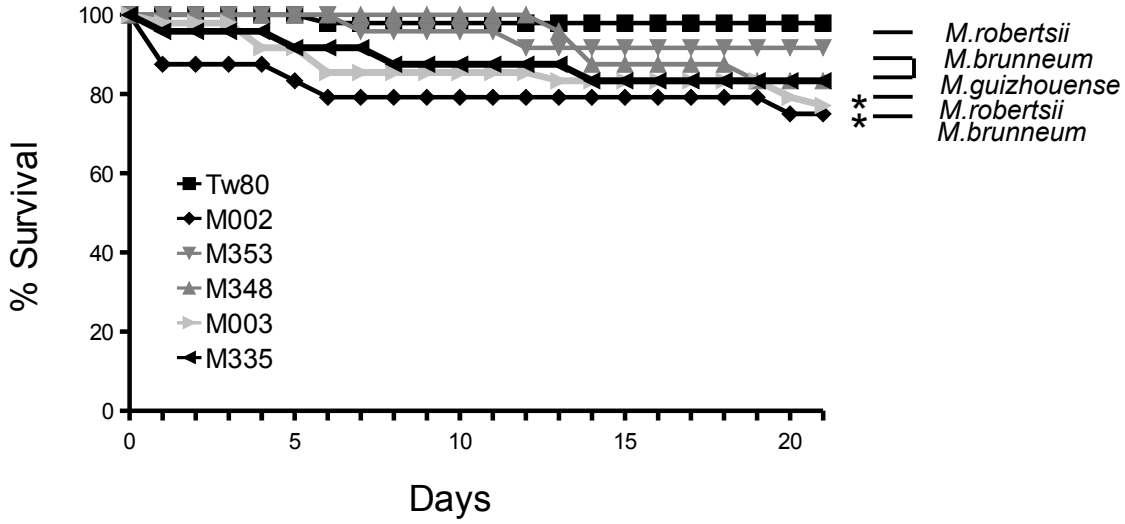
Figure 4. Cox regression analysis of survivorship of worker termites from four termite colonies challenged with six fungal isolates of *Metarhizium* (10^7 conidia mL^{-1}) over the course of 21 days. Significant differences in survivorship compared to controls are indicated with asterisks (* $p < 0.05$, ** $p < 0.001$). Species names are given to the right of each graph.



4 C)


353 Termite Survival
% Survival
Days
Tw80
M002
M353
M348
M003
M335
MGW
M.guizhouense
M.robertsii
M.brunneum
M.robertsii
M.brunneum

4 D)


GF2 Termite Survival
% Survival
Days
Tw80
M002
M353
M348
M003
M335
M.robertsii
M.brunneum
M.guizhouense
M.robertsii
M.brunneum

The *Metarhizium* isolates also varied in their virulence and this variation clearly depended on the treatment colony so a highly virulent isolate in one colony could be benign in another colony (Figure 4). For example, among the two colonies that showed greater overall vulnerability to infection (335 and 003), *Metarhizium* isolate M348 showed significant virulence in colony 335 (Wald = 23.21, df = 1, $p < 0.001$) but not in colony 003 (Wald = 1.41, df = 1, p = 0.235).

To investigate the relationship between the genetic identity of the different isolates and their virulence in different colonies, we tested for a correlation between the genetic distance between *Metarhizium* isolates and the difference in virulence between the isolates. A significant correlation is expected if isolates with similar levels of virulence are genetically similar. Virulence was measured with the hazards ratios of death of *Metarhizium* treatments compared to controls calculated with the Cox regression analysis. The correlation and associated p-values are shown in Table 2. All colonies with the exception of 002 showed a significant correlation. The highest correlation (r = 0.77) occurred in colony 003, which appeared to have the greatest variation in susceptibility to the different *Metarhizium* isolates (Figure 4).

Colony	Correlation (r)	P-value
335	0.770	0.002
003	0.550	0.045
353	0.586	0.030
GF2	0.354	0.164

Table 2. Correlations between differences in genetic distance and hazard ratios of survivorship for different *Metarhizium* isolates in four colonies of *R. flavipes*.

There was no significant correlation between physical and genetic distance for the different *Metarhizium* strains used in this study (zt test, r = 0.186, p = 0.256). Colonies did not show a difference in susceptibility to strains of *Metarhizium* collected in the immediate vicinity of the colony compared to strains that were not in close proximity to the colonies (Wald = 1.645, d.f. = 1, p = 0.2).

Discussion

The diversity of *Metarhizium* isolates over the small spatial scale investigated in this study is strikingly high. Three separate species were identified within a 1 km transect, *M. brunneum*, *M. robertsii* and *M. guizhouense*. *Metarhizium anisopliae* has recently been divided into different species based on geneological concordance determined with a multilocus phylogeny (Bischoff *et al.,* 2009). This method, also known as Phylogenetic Species Recognition (PSR), identifies the most recent taxonomic unit in which recombination is not occurring between two or more genes (Taylor *et al.,* 2000). This is useful approach for identifying *Metarhizium* species because species of this Ascomycete cannot be readily identified with morphological characteristics.

There is a correlation between genetic distance among fungal isolates and their difference in virulence. The genetic identity of an isolate determines its virulence (Figure 4) and the genetic identity of colony may affect its susceptibility. The susceptibility of termite colony 335, 003 and 353 to the *Metarhizium* isolates corresponded with the species identification as defined by PSR (Figure 4). The susceptibility of termite colony 002 to the *Metarhizium* isolates did not correspond with the species identifications. However, workers in this colony were resistant to infection and only two isolates (M002 and M003) showed significant mortality over the course of the experiment.

Increased resistance to infection may be attributable in part to genetic characteristics of different colonies such as levels of inbreeding (Vargo and Husseneder, 2012) or variation in the expression level of immune genes. Variation in expression levels of β-1,3-glucanases (GNBP2) have been observed among workers from different *R. flavipes* colonies that were exposed to *M. anisopliae,* with a range of 0.65 to 12.14 fold increase in expression levels among eight colonies compared to untreated controls (Gao *et al.,* 2012).

The appearance of novel *Metarhizium* strains, which are particularly virulent in termite hosts, as a result of random mutation could cause periodic epizootics in *Reticulitermes*. This suggests that among *Metarhizium* strains in this study, *M. robertsii* was most likely to have caused a recent epizootic because it is the most common isolate,

represented in 11of 15 soil samples collected adjacent to foraging workers (Figure 2). *M. guizhouense* and *M. brunneum* were only identified in one and three soil samples, respectively. This study represents a snapshot of *Metarhizium* strain diversity. Repeated sampling of different sites might help reveal if *Metarhizium* strains vary in their prevalence, which would provide support for the occurrence of periodic epizootics.

The variation in isolates seen at small spatial scales could drive adaptive evolution in the termite immune system. Although this study represents a snapshot of *Metarhizium* diversity that does not capture the dynamics of change over time, it suggests that *Metarhizium* could drive adaptive evolution in the termite immune system. Selective sweeps of components of the innate immune system such as termicins and GNBPs may arise as a result a virulent *Metarhizium* isolate spreading among colonies, countered by the evolution of termite resistance to that isolate.

Chapter 3

A comparison of the selective pressure on antifungal genes in *Cryptocercus* woodroaches and termites

Abstract

Messenger RNA sequences of Gram Negative Bacteria-binding Protein 1 (*GNBP1*) were identified and analyzed in two species of subterranean termites. Using population genetic methods, we made comparisons between this gene and two additional antifungal genes in the subterranean termites and two species of woodroaches, *Cryptocercus punctulatus* and *Cryptocercus wrighti*. An analysis of nucleotide intraspecific polymorphism indicates that these genes frequently face selective sweeps, possibly as a result of a virulent fungal strain spreading through populations and selecting for resistant specific alleles that afford the greatest resistance to infection.

Introduction

The selective pressure imposed by pathogens on the immune system is expected to be especially acute in social insects such as termites with long-lived and crowded colonies of closely related kin (Schmid-Hempel, 1998). Molecular evolutionary studies of some of the effectors of immune defense in termites indicate that they face strong selective pressure to change, which may be a response to pathogens trying to usurp or evade the immune system (Bulmer and Crozier, 2006). Termite antifungal proteins including defensins (*termicins*) and β-1,3-glucanases (Gram Negative Bacteria-binding Proteins or GNBPs) may be under unusually strong selective pressure relative to defensins and *GNBPs* in other insects because they have been co-opted from an internal role in the innate immune system to an external role in a social immune defense strategy (Bulmer *et al.,* 2009). The external secretion of these antifungals pre-emptively kills fungal pathogens such as *Metarhizium anisopliae,* which can evade the immune system once it has entered the hemocoel. The effectiveness of this external defense strategy depends on social behaviors such as grooming, cannibalism, necrophagy and burying of corpses (Chouvenc *et al.,* 2008; Chouvenc *et al.,* 2010).

Termites apparently depend on *GNBPs* and *termicins* for antifungal defense as inhibition or suppression of these proteins increases the susceptibility of termites to fatal infections by *M. anisopliae* (Bulmer *et al.,* 2009). Inhibition of GNBP2 β-1,3-glucanase activity with glucono delta lactone (GDL) significantly increases the susceptibility of *Nasutitermes corniger* workers to a laboratory strain of *M. anisopliae* (Bulmer *et al.* 2009) and *Reticulitermes flavipes* workers to naturally encountered strains of *M. anisopliae* (Hamilton *et al.* 2011). RNA interference (RNAi) experiments provide evidence that the GNBP2 and termicin are critical for *Reticulitermes* defense against *M. anisopliae*. Ingestion of double stranded RNA corresponding with a small region of *termicin* or *GNBP2* gene sequence increases *R. flavipes* worker susceptibility to local strains of *M. anisopliae* (Hamilton and Bulmer, 2012).

Termites employ hygienic behavioral strategies for defense against *M. anisopliae* and the strategies are likely to depend on or are augmented by termicins and GNBPs. The

β -1,3-glucanase activity of GNBPs has been identified in the masticated and manipulated materials used to bury corpses (Bulmer *et al.* 2009, Hamilton and Bulmer, 2011). This enzyme activity has also been identified on the insect cuticle (Hamilton and Bulmer, 2011, 2012), suggesting that it is spread over external surfaces with allogrooming. β-1,3-glucanase activity associated with GNBPs and possibly additional enzymes produced by protists has been identified in the hindgut where it may be instrumental in inactivating ingested conidia and infected corpses (Bulmer *et al.* 2009, 2012). Allogrooming in termites increases after exposure to *M. anisopliae* conidia (Rosengaus *et al*. 1998), which suggests that the active dissemination of antifungals helps limit epizootics. This contrasts with an antifungal strategy employed in the *Lasius* ants in which allogrooming helps inoculate nestmates (Konrad *et al.* 2012). Low concentrations of conidia spread by allogrooming prime individual innate immune defenses and protection from an epizootic does not appear to depend on the collective dissemination of secreted antifungals.

The selective stress on the termicin/GNBP system may reflect termites' vulnerability to fungal pathogens due to their complex social structure. However, this antifungal system appears to have evolved prior to the evolution of the termites and in response to physiological stress that was not necessarily attributable to crowded colonies (Bell *et al.* 2007). Termites evolved from a cockroach-like ancestor that is likely to have shared subsocial characteristics with woodroaches belonging to *Cryptocercus*, which represent the closest living relatives to termites (Lo *et al.* 2000; Inward *et al.* 2007; Engel *et al.* 2009; Ohkuma *et al.* 2009). These insects have a nitrogen poor diet and avoid heavy nitrogenous investment in the development of the external cuticle until late developmental stages (Bell *et al.* 2007; Nalepa 2011). Prolonged development of four or more years (Bell *et al.* 2007) with a thin cuticle is likely to increase a nymph's vulnerability to fungi that can rapidly grow through thin cuticle. The termicin/GNBP antifungal defense may have evolved to accommodate this increased vulnerability.

Antifungal β-1,3-glucanase activity associated with secreted GNBPs has been identified in *C. punctulatus* (Bulmer *et al.* 2012). In this study, we identify *GNBP1* in

Reticulitermes flavipes and *Reticulitermes virginicus* and use a population genetics approach to investigate whether GNBPs and termicins in these species face similar selective pressure to the orthologous proteins in woodroaches.

Materials & Methods

Insect and fungus sample preparation

R. flavipes and *R. virginicus* were collected along a transect of approximately 2 kilometers close to the intersection between Gun Powder Falls River and Route 1, Maryland, USA, during July and August of 2009. *R. flavipes* and *R. virginicus* collection points were separated by at least 90 and 140 meters, respectively, which are distances that ensure that most collections represent separate colonies based on previous measures of foraging range in *Reticulitermes* (Vargo and Husseneder 2009). Both workers and soldiers were collected from small pieces of wood lying on the soil or as they moved between soil and wood.

Nymphs and adults of the woodroach *C. punctulatus* were collected from decayed wood approximately 2 miles west of Huntly, Virginia, in November 2010. Nymphs and adults of the woodroach *C. wrightii* were collected from decayed wood located in Dark Horse Hollow picnic area, Virginia, in November 2010. Termites that were collected had the potential to contain multiple reproductives and several generations of offspring while woodroach collections consisted of a single set of parents and typically a single generation of nymphs.

Termites and woodroaches were maintained with pieces of wood in plastic containers at 25°C in constant dark and kept moist by spraying with water once a week. Insects from each collection point were gently crushed in RNA later (Sigma-Aldrich) and stored at –20°C. The mRNA was prepared by removing excess RNA later with centrifugation (8000g, 1min) and purified with a Quick Prep mRNA kit (Amersham Biosciences). In addition, insects were stored for DNA preparation at –20°C in 100% ethanol. DNA was prepared with a Qiagen DNA purification kit.

The termites had been identified previously with taxonomic keys, geographical location and comparisons of partial mitochondrial 16S rRNA with sequences deposited in GenBank (Bulmer and Crozier, 2006; Bulmer *et al.*, 2009; Bulmer *et al.*, 2010). The identity of the woodroaches was verified with morphological characters, geographical

location and comparisons of 16S rRNA with sequences in GenBank. 16S rRNA was sequenced using the protocol and primers described in (Ye *et al.*, 2004).

Local fungal strains of *M. anisopliae* were isolated from soil samples taken from nearby each termite and woodroach collection site and the soil was free of termites or woodroaches. The baiting technique involved placing five *Tenebrio molitor* larvae, which are highly susceptibility to infection by the fungus, in to 50 g of soil and monitoring for mealworm deaths. Bodies were then surface sterilized with 70% ethanol and set aside in petri dishes with moist filter paper for a week before being checked for confirmation of *M. anisopliae* infection. Conidia were collected from individuals that died of infection with a sterile inoculation loop, streaked on potato dextrose agar and grown at 25 ºC to obtain single clonal strains that were further cultivated to produce conidia. The identity of *M. anisopliae* var. anisopliae was confirmed by comparing the ITS region, amplified with the universal primers PN3 and PN34, with GenBank sequences (Viaud *et al.*, 2000).

Isolation & characterization of GNBP1

The full mRNA transcripts for *GNBP1*s from different *Reticulitermes* collection points were amplified with the protocol and primers described previously (Bulmer and Crozier 2004). PCR products were cloned into the PGEM-T Easy Vector system (Promega) and one to three clones with the correct insert were sequenced for each collection point.

Statistical analysis

RNA transcripts were aligned manually with Se-Al, version 2.0a11 (Rambaut 2002). The aligned sequences were analyzed with the program DnaSP, version 5.1 (Rozas and Rozas 1999).

Results

GNBP1 was successfully identified and sequenced in *R. flavipes* and *R. virginicus*. An alignment or 1071 nucleotides was used in the analysis. The termite *GNBP1* sequence was compared with other published *termicin* and *GNBP2* sequence (Bulmer *et al.*, 2010) and unpublished *termicin*, *GNBP1* and *GNBP2* sequence from *C. punctulatus* and *C. wrightii*. For *Cryptocercus*, the *termicin* open reading frame (ORF) was 183 nucleotides. The *termicin* transcripts included a 3'untranslated region (*3'UTR*), which was 298 nucleotides in length without inclusion of the poly-A tail. The *GNBP2* ORF was 1086 nucleotides.

Tajima's *D* (Tajima 1983) was used to detect evidence of selective sweeps or balancing selection acting on *termicin* and the *GNBPs* in each species of *Reticulitermes* and *Cryptocercus*. Tajima's *D* compares the proportion of nucleotide pairwise mismatches with the number of segregating sites and is expected to be ~0 if mutation and genetic drift alone determine the frequency distribution of pairwise mismatches and segregating sites. Balancing selection creates an excess of pairwise mismatches relative to polymorphic sites so that Tajima's *D* is likely to be positive whereas selective sweeps reduce the frequency of mismatches relative to polymorphic sites so that Tajima's *D* is likely to be negative.

The results for Tajima's *D* (Tajima, 1983) are shown in Table 1. Tajima's *D* (Tajima, 1983) was significantly lower than zero for *GNBP2* in both *C. punctulatus* and *C. wrighti* but not for *GNBP1* in either woodroach species (Table 1). However, Tajima's *D* was significantly lower than zero for *GNBP1* in both *R. flavipes* and *R. virginicus* but not for *GNBP2* in either termite species (Table 1). Tajima's *D* was significantly lower than zero for *termicins* in both *R. flavipes*, *R. virginicus* and *C. punctulatus* but not for *C. wrightii* (Table 1).

The significant negative values suggest that in the past selection favored specific alleles and swept away other variants (Tajima's *D* detects the recovery from a selective sweep towards neutral patterns of nucleotide variation rather than the sweep itself).

GNBP1	sample size	Tajima's *D*	p-value
C. punctulatus	8	-0.723	p=0.261
C. wrighti	13	-0.287	p=0.395
R. flavipes	17	-1.394	p=0.039*
R. virginicus	18	-1.533	p=0.024*
GNBP2			
C. punctulatus	10	-2.091	p<0.001***
C. wrighti	10	-1.925	p=0.003**
R. flavipes	19	-0.911	p=0.073
R. virginicus	17	-0.599	p=0.188
termicin			
C. punctulatus	19	-2.110	p<0.001***
C. wrighti	21	-0.810	p=0.230
R. flavipes	15	-1.673	p=0.011*
R. virginicus	13	-1.508	p=0.023*

Table 1. Measures of Tajima's *D* for *GNBP1*, *GNBP2* and *termicin*. Sample size corresponds with the number of sequenced clones for each gene. The p-values were calculated with 10,000 coalescent simulations.

The McDonald-Kreitman (MK) test was used to compare polymorphism and divergence at nonsynonymous and silent sites (McDonald and Kreitman 1991). The ratio of nonsynonymous to silent fixed differences between species will be similar to the ratio of nonsynonymous to silent polymorphism within species if mutation and divergence operate under the standard neutral model. A significant excess of nonsynonymous substitutions between species provides evidence that positive selection rather than genetic drift has driven sequence divergence.

We could not implement the MK test for *GNBP1* or *termicin* in *Cryptocercus* because there was a complete lack of either nonsynonymous or synonymous fixed differences. These two species are likely to have diverged recently and selection or genetic drift has apparently not had sufficient time to fix nucleotide differences between *GNBP1s* and *termicins* in the two species.

The McDonald-Kreitman test of *GNBP2* in *Cryptocercus* was significant because of an excess of synonymous to nonsynonymous fixed sites compared to synonymous to nonsynonymous polymorphic sites (Table 2). Together with the results from Tajima's *D*, a

complete lack of nonsynonymous fixed differences between the *GNBP2s* suggests that a phenotypically identical allele (no fixed nonsynonymous differences) that differed at 11 synonymous sites swept through the two species. Selection has apparently driven the fixation of these 11 synonymous nucleotides because they were hitchhiking with an adaptive allele.

	Synonymous sites, Fixed/Polymorphic	Nonsynonymous sites, Fixed/Polymorphic	Fisher's exact test p-value (two tailed)
GNBP1	0/21	0/16	NA
GNBP2	11/14	0/19	p<0.001
termicin	0/15	0/7	NA

Table 2. McDonald-Kreitman test of *GNBP1, GNBP2* and *termicin* in *Cryptocercus*. A total of 20 sequences (*C. punctulatus*, n=10; *C. wrighti*, n=10) were used in the analysis of the *GNBPs* and 40 sequences (*C. punctulatus*, n=19; *C. wrighti*, n=21) were used in the analysis of the *termicins*.

The MK test of *termicins* but not *GNBPs* was significant in *Reticulitermes* (Table 3). There has been a significant excess of *termicin* nonsynonymous substitutions between species, which provides evidence that positive selection has directed amino acid changes.

	Synonymous sites, Fixed/Polymorphic	Nonsynonymous sites, Fixed/Polymorphic	Fisher's exact test p-value (two tailed)
GNBP1	1/11	3/12	p=0.603
GNBP2	2/19	7/25	p=0.291
termicin	12/18	15/3	P=0.002

Table 3. McDonald-Kreitman test of *GNBP1, GNBP2* and *termicin* in *Reticulitermes*. A total of 35 sequences (*R. flavipes*, n=17; *R. virginicus*, n=18) were used in the analysis of the *GNBP1*, 36 sequences (*R. flavipes*, n=19; *R. virginicus*, n=17) were used in the analysis of the *GNBP2* and 28 sequences (*C. puntulatus*, n=15; *C. wrighti*, n=13) were used in the analysis of the *termicins*. The results for the termicins have been published previously (Bulmer et al. 2010).

Discussion

Our results suggest that antifungal proteins in both termites and woodroaches face selective pressure that has resulted in selective sweeps favoring one or a few alleles. However, different *GNBPs* show evidence of selective sweeps in the termites and woodroaches. *GNBP2* has been shown to be important for antifungal defense in the termites and woodroaches (Bulmer *et al*. 2009). The selective sweep of *GNBP1* in the termites suggests that it may also play an antifungal role, at least in termites, especially given Tajima's *Ds* are very similar for the termite *GNBP1s* and *termicins*, which has a known antifungal role (Bulmer *et al.* 2009; Hamilton *et al.* 2011, Lamberty *et al.,* 2001). The MK test suggests that the selective sweep in the termite *termicins* was driven by positive selection of an adaptive allele. The MK test did not detect positive selection in termite *GNBP1s*. However, the MK test for the termite *GNBPs* may lack power because of the limited number of nucleotide polymorphism and fixed differences.

Demographic expansion or contraction can also influence Tajima's D and lead to a deviation from zero associated with neutral evolution. However, demographic changes would have the same effect on both *GNBP1* and *GNBP2* and therefore are unlikely to have contributed to the significantly different measures of Tajima's *D*. Instead selective pressure appears to have differed between the two genes, possibly because they have different functional roles in immunity and/or digestion.

Both species of wood roach may have recently faced a common fungal epidemic that selected for a phenotypically identical *GNBP2* allelic variant. The two insect species are physically isolated from each other; the insects are wingless and are found at relatively high altitudes (>500 meters) on separate ridges of the Appalachian Mountains in Virginia. However, the conidia of fungal pathogens such as *Metarhizium* could easily cross this divide when carried by wind or other insect hosts. These recently diverged species may therefore have encountered the same pathogen, which selected for an identical antifungal gene variant.

There are similarities between woodroaches and termites such as their thin cuticle that make both types of organisms susceptible to fungal pathogens including *Metarhizium*. A thin cuticle is not shared with other social insects such as most ants and

therefore, *Metarhizium* isn't as effective and results in limited mortality in those species (Bot *et al.,* 2002). Fungal epizootics occur when a pathogenic microbe, such as *Metarhizium*, exploits a susceptible host and disperses throughout the host population (Chouvenc *et al.,* 2010). While these epizootics are limited in termite colonies, selective pressure on antifungal proteins may arise due to periodic epizootics from a virulent *Metarhizium* strain. The diversity of strains encountered by termites and possibly woodroaches that vary in their virulence suggests that these insects will periodically encounter a virulent strain that results in an epizootic (Chapter 2).

Future research should include further investigation of termite and woodroach *GNBPs* and will include different *Cryptocercus* species from other sites in the Appalachian mountains. Analysis of their *GNBPs* and *termicins* would provide further knowledge on the selective pressures that the antifungal proteins face in their natural environment.

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Summary

I am a recent molecular and cell biology Masters graduate from Towson University and I am excited to explore opportunities within the field of cancer or genetics research. For the past three years I have been an active member of an entomology research lab, under the direction of Dr. Mark Bulmer, in which I am learned basic molecular techniques and analysis to investigate termites innate immune system. I have applied what I have learned to my Masters Thesis by looking at strain variation of the fungal pathogen *Metarhizium* and how termites, specifically the species *Reticulitermes flavipies,* vary in susceptibility among different fungal strains.

Education

Masters of Science in Molecular and Cell Biology — Graduation:
Towson University, Towson, MD — January 6th, 2013
Course Work: Applied Biotechnology, Data Interpretation, Evolutionary & Ecological Physiology, Gene Expression, Membrane Biology, Molecular Neuroscience Seminar,; **Lab Courses** – Immunology

Bachelor of Science in Cell and Molecular Biology — May 2010
Towson University, Towson, MD
Course Work: Animal Physiology, Biodiversity, Calc Based Physics 1&2, Cell Biology, Endocrinology, Virology; **Lab Courses** – Genetics, Microbiology, Molecular Biology

Associate of Science in Biology — December 2008
Frederick Community College, Frederick, MD
Course Work: Calculus 1, 2&3; **Lab Courses** – Biology 1&2, Chemistry 1&2, Forensic Biology, Organic Chemistry 1&2

Skills

Laboratory Techniques:

Proficient in: Pipetting small volumes, Producing sterilized media plates and basic sterile techniques (Autoclave), Plating single colonies, Cell morphology, PCR, PCR sequence cleanup, Making acrylamide and agarose gels, Gel Electrophoresis analysis, Filter centrifugation, gel purification of DNA fragments, Restriction digests, DNA sequencing, Mass Spectroscopy (NanoDrop), Survival assays and LD50.

Knowledge of: RTPCR, bacterial transformation, and plasmid purification

Computer skills:

Adobe Photoshop, Adobe InDesign, Apple, JMP/SAS, Linux, Open Office, Manual Sequence Alignment (SeAL), Microsoft Windows, Microsoft Office - Excel, Powerpoint, and Word

Research experience

Laboratory Research Assistant — Summer 2009 – Jan 2013
Under Direction of Mark Bulmer, Ph.D
Towson University, Towson, MD

Research in this laboratory led to the completion of my thesis called "Antifungal defenses in subterranean termites and *Cryptocercus* woodroaches." Research experience includes field collection and maintenance of termites, spore baiting techniques, gel electrophoresis, production of PDA/Amp plates, PCR, PCR product clean up, agarose gel, SDS page gel, poly-acrylamide gel, protein purification (specifically GNBP), sequence alignment (SeAl), statistical analysis (JMP/SAS).

Teaching experience

Teaching Assistant (TA) – Biology for Non-majors — Fall 2010 – Dec 2012
Towson University, Towson, MD

Introduce necessary background information for students to understand and successfully complete all labs. **Fall 2012 -** Sole responsibility for three stand alone laboratory sections of Biol 120 Lab. Weekly grading of lab reports using online system (Blackboard) with feedback given to approximately 72 students in a timely fashion. **Spring 2011 – Spring 2012 -** Responsible for three laboratory sections of Biol 115 lab that was paired with the lecture course. **Fall 2010 -** Responsible for one laboratory section and split for team teach of second laboratory section. Grading of lab notebooks and group projects resulting in 25% of students Biology lecture grade. Additional assistance given by grading for two lecture sections comprising of over 170 students.

Presentations

IUSSI-NAS Conference — October 5-7, 2012
Greensboro, NC

As a member of the IUSSI-NAS, I was invited to present my previous research from Mark Bulmer's lab at Towson University at the Social Insect conference. The presentation included a poster titled: "A common antifungal defense strategy in *Cryptocercus* woodroaches and termites." Our research introduced the idea that Cryptocercus and termites rely on similar GNBP's to protect them from pathogenic microbes and fungus, such as *Metarhizium anisopliae*.

Publications

"A common antifungal defense strategy in *Cryptocercus* woodroaches and termites"

Journal: Insectes Sociaux, Accepted: May 18, 2012, Currently In Press

Authors: Mark Bulmer, Diandra Denier, Joe Velenovsky, Casey Hamilton

Abstract: We identified and characterized Gram-negative bacteria binding proteins (GNBPs) and their predicted antifungal activity in the woodroach *Cryptocercus punctulatus*. *C. punctulatus* is likely to share many characteristics with the subsocial ancestor of the Isoptera, including allogrooming, which facilitated the evolution of termite sociality. Based on a phylogenetic analysis, an ancestral GNBP with an intact b-1,3-glucanase active site appears to have duplicated in a common ancestor of subsocial Cryptocercus woodroaches and termites. In termites, the secreted b-1,3-glucanase activity of GNBPs provides important prophylactic protection from fungal pathogens such as *Metarhizium anisopliae*, which can evade the immune system after entering the insect. Here, we identify b-1,3-glucanase activity on the cuticular surface of *C. punctulatus* that originates from the salivary gland and is likely spread by allogrooming. Cuticular washes have antifungal activity against *M. anisopliae* conidia that is suppressed by an inhibitor (GDL) of termite GNBP b-1,3-glucanase activity. *C. punctulatus* nymphs that are treated with GDL and subsequently exposed to *M. anisopliae* conidia show significantly greater mortality than the untreated nymphs exposed to conidia. A molecular evolutionary analysis of GNBPs in two species of *Parcoblatta, Periplaneta americana, C.punctulatus* and representative termites indicates that selection-directed change in a glycosylphosphatidylinositol (GPI) anchor region. Modification of the GPI anchor region may have been instrumental in the evolution of an antifungal defense strategy that depends on the external secretion of GNBPs from the salivary gland and their dissemination by grooming. This strategy may have helped compensate for the vulnerability of a subsocial woodroach-like ancestor to fungal disease that results from prolonged development with a thin cuticle and facilitated the transition to termite eusociality.

Volunteer experience

Co-Captain Science Against Cancer Team Relay for Life — Spring '10, '11, '12

Towson University, Towson, MD

Organized Women in Science, Tri Beta, MB3, Minorities in Science, SACS, and Graduate student involvement in Towson's Relay for Life event on April 20, 2012. Represented the Science Against Cancer at Team Captain meetings and came up with fund-raising ideas including ordering team t-shirts and designing team logo for embroidery. Trained new team captain to take over when I graduate to ensure the teams success in future years. My team raised over $1,500 to support cancer research.

Organized both Women in Science, Tri Beta, and MB3 as well as Graduate student involvement in Towson's Relay for Life event on March 8, 2011 and my team raised over $2,500. Team Captain for Women in Science and Tri Beta at Towson's Relay for Life event on March 26, 2010 and my team raised over $1,000 to support cancer research.

Organizations

Member of IUSSI-NAS — Fall 2011 – Fall 2012
International Union for the Study of Social Insects
North American Section

Active Member of National Biological Honor Society — Spring 2009 – Fall 2012
Tri Beta - Upsilon Eta Chapter
Towson University, Towson, MD

Member of Towson's Women In Science Club — Fall 2008 – Fall 2012
Towson University, Towson, MD

Member of Chestnut Burr Yearbook Staff — Fall 2003 – Spring 2006
Middletown High School, Middletown, MD

Worked in the layout department of the yearbook staff for three years. Designed overall look of yearbook pages using Adobe InDesign and Adobe Photoshop. Techniques maintained while producing Powerpoint presentations using Microsoft Office for teaching purposes and class presentations.

References – Towson University

Mark Bulmer, Ph.D, Research Advisor for Thesis work: mbulmer@towson.edu

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