

## ABSTRACT

CALDWELL, NATHAN DAVID. Investigating Biopesticides for Red Imported Fire Ant (*Solenopsis invicta* Buren): Efficacy and Behavioral Modifications Associated with Entomopathogens. (Under the direction of D. Wes Watson and Charles S. Apperson).

The pathogenicity and virulence of foreign and domestically isolated entomopathogens against monogyne and polygyne social forms of *Solenopsis invicta* workers was explored. In a laboratory study, ants were exposed to three strains of *Beauveria bassiana* and three strains of *Metarhizium* spp. that were originally isolated from fire ants within Brazil or various insects throughout the USA. Doses applied ranged from  $7.6 \times 10^6$  to  $3.4 \times 10^7$  conidia per mg of dry material. Mortality was significantly higher in fire ants exposed to all *Beauveria* strains (100%) than to *Metarhizium* strains ( $53.7\% \pm 15.4\%$ ). No statistical difference in cumulative mortality was detected between social forms of fire ants at 9 d post-exposure. Results indicate that *Beauveria* strains originally isolated from house flies in the United States are highly virulent to *S. invicta* workers.

Parasitic infection often induces behavioral alterations in the host which may ultimately benefit the host or the parasite; however, whether these behavioral changes are mechanisms of host defense, manipulations by the parasite or coincidental events is not always clear. Infected ants had no apparent phototactic response during the infection process. A mark and recapture bioassay evaluated the movement of fire ants during the infection period and showed that erratic movement and elevation seeking began on day 2 and peaked on day 4. Average time between marking a climbing ant and subsequent death was 1.32 d. In a heat gradient assay, the majority of control ants ( $86 \pm 13\%$ ) were aggregated within the  $< 27^\circ\text{C}$  zone and remained there for the entirety of the 5 day assay. An average of 47% and 31% of the treated ants were located at temperatures  $> 27^\circ\text{C}$  on day 2 and 3,

respectively. Based on lack of survival in the heat gradient choice assay and the dispersion and movement upwards during late infection period, it is suggested that the alteration in normal host behavior is a benefit to *B. bassiana* and increases opportunities for dissemination.

Horizontal transmission of conidial propagules from exposed workers to unexposed nestmates during grooming events and subsequent mortality was studied. Workers were exposed to conidia and allowed to self-groom only, or amongst a small cohort of nestmates. After 4 h, exposed ants that were in multiple worker experiments had a 31.3% reduction in conidia washed from the cuticle compared to self-grooming alone. All ants directly exposed to conidia died. Originally unexposed ants, introduced to a conidia-exposed nestmate for 4 h had mortality rates equivalent to the negative controls. Reduced transmission from exposed to unexposed ants was not detected at other time intervals. It is hypothesized that an increased but limited duration of consistent exposure to conidia-exposed nestmates enhances allo-grooming behaviors and chemical defense mechanisms which results in reduced mortality.

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Investigating Biopesticides for Red Imported Fire Ant (*Solenopsis invicta* Buren):  
Efficacy and Behavioral Modifications Associated with Entomopathogens

by  
Nathan David Caldwell

A thesis submitted to the Graduate Faculty of  
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## **DEDICATION**

To my endlessly supportive wife, Kelly; thank you for your love and encouragement.

## **BIOGRAPHY**

Nathan David Caldwell was born and raised in Indianapolis, Indiana to Randall and Sherry Caldwell. He attended Lincoln Memorial University where he completed a Bachelors of Science in Wildlife and Fisheries Management in 2000. Subsequently, he worked for Dr. Craig Reinemeyer as a laboratory and field parasitology technician at Eastern Tennessee Clinical Research prior to beginning his Master's Degree at The University of Tennessee in 2002. Upon completion of his M.S. degree in Entomology and Plant Pathology in 2004, Nathan began to pursue a Ph.D. in the Department of Entomology at North Carolina State University. In 2010, he began his career with FMC Corporation as a Research Entomologist at the Sparks Research Station in Sparks, Georgia and subsequently transferred to the Ewing, New Jersey Agricultural Solutions division in order to conduct early stage research and development testing as a Research Biologist in 2013.

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## CHAPTER 1

Caldwell: Efficacy of *Beauveria* and  
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**Efficacy of *Beauveria* and *Metarhizium* strains isolated from indigenous and foreign locales against monogyne and polygyne social forms of the red imported fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae)**

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**Abstract.** The invasion of the red imported fire ant (*Solenopsis invicta* Buren) into the United States has had medically, economically, and ecologically significant impacts. Based upon temperature and precipitation data models and the increased international transportation of agricultural materials, the risk and potential of the red imported fire ant to expand globally is likely. Effective natural microbial pesticides could enable the supplementation of chemical treatments with virulent, biological alternatives. The biological control provided by strains of *Beauveria* and *Metarhizium* are well documented against fire ants; however, the relative pathogenicity and virulence of foreign and domestically isolated strains against monogyne and polygyne social forms of *S. invicta* workers is unexplored. We exposed ants to undiluted *B. bassiana* conidial concentrations of  $2.2 \times 10^7$  (BbP89),  $3.4 \times 10^7$  (BbL90),  $1.7 \times 10^7$  (Bb447) per mg of dry material; *Metarhizium robertsii* (Ma2561), *M. brunneum* (Ma3738), and an uncataloged *Metarhizium* strain (MaNC) isolated from a cockroach were applied at concentrations of  $7.6 \times 10^6$ ,  $9.1 \times 10^6$ , and  $1.1 \times 10^7$  conidia per mg of dry material, respectively. Strains Bb447, Ma2561, and Ma3738 were isolated from fire ants. Strains BbP89 and BbL90 were originally isolated from house flies (*Musca domestica* L.). Mortality was significantly higher in fire ants exposed to all *Beauveria* strains (100%) than to *Metarhizium* strains ( $53.7\% \pm 15.4\%$ ). Overall susceptibility to infection was not affected by social form. Results indicate that strains originally isolated from house flies in the United States are highly virulent to *S. invicta* workers. Average mortality was influenced by *Metarhizium* isolate with reduced mortality at 9 d to ants exposed to the MaNC strain ( $33.1\% \pm 6.8\%$ ). In a dose-response experiment utilizing *Beauveria bassiana* strain Bb447, the

infection period was extended over a longer period of time when monogyne and polygyne ants were exposed to the low dilution and resulted in statistically reduced mortality at 4 d.

**Keywords.** Fire ant, *Solenopsis*, social form, *Beauveria*, *Metarhizium*, biological control, entomopathogen

## Introduction

The red imported fire ant, *Solenopsis invicta* Buren, was introduced into the United States through the port of Mobile, AL via shipping from South America in the late 1930's or early 1940's (Buren 1972). *Solenopsis invicta* in the southern United States likely came from one point of origin located in the Mesopotamia flood plain near Formosa, Argentina and was not the product of multiple invasions (Caldera et al. 2008). Over the next 75 years fire ants have become more prevalent in the United States and colonies approximately 40% larger and four times as dense compared to infested locales in South America (Porter et al. 1992, 1997). This observed expansion was consistent with the theory of introductions into a location where relatively few natural enemies were encountered (Jouvenaz et al. 1977). *Solenopsis* in South America is host to a minimum of 32 natural enemies including microorganisms, nematodes, parasitic phorid flies, and a parasitic ant (Porter et al. 1997, Tschinkel 2006) which provides opportunities for the assessment and use of biological control techniques in the United States. Within the United States, range expansion northward is impacted by freezing temperatures (James et al. 2002); however, based upon temperature and precipitation data models and the increased international transportation of agricultural materials, the risk and potential of the RIFA to expand globally is likely (Morrison et al. 2004, Ascunce et al. 2011, Wetterer 2013, Bertelsmeier et al. 2015).

Chemical insecticides remain an integral component to an integrated pest management (IPM) approach for the control of *S. invicta* (Williams et al. 1999), but the continued assessment and further evaluation of natural microbial pesticides could enable the supplementation of chemical treatments with virulent, biological alternatives (Vega et al.

2012). Indigenous pathogens, including the soil fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin, have been isolated from *S. invicta* workers in Brazil (Allen and Buren 1974, Stimac et al. 1987, Alves et al. 1988, Pereira et al. 1993, Oi et al. 1994). Several studies have evaluated the fungus *B. bassiana* in the control of *S. invicta* which resulted in decreased populations in laboratory and field assays with varying and inconsistent success (Siebeneicher et al. 1992, Pereira et al. 1993, Stimac et al. 1993a, b). The fungus *M. anisopliae*, the causal agent of green muscardine diseases of insects, was recognized as a potential biocontrol agent in 1879 by Metschnikoff, and has been studied in the control of numerous agricultural and structural pest species (Zimmermann 1993). In 1992, *M. anisopliae* was detected in 0.5% of founding *S. invicta* queens collected in Texas (Bextine and Thorvilson 2002).

The fungal pathogen *M. anisopliae*, like *B. bassiana*, is a species that reproduces asexually. Like many fungi, these species penetrate a host's epicuticle with a flattened and thickened tip of a hyphal branch (appressorium) that produces specialized penetration structures, such as germ tubes and penetrant hyphae, also known as infection pegs (Boucias and Pendland 1991, Hajek and Leger 1994). Once the epicuticle is breached, progress by the penetration peg through the procuticle may continue inward via penetrant hyphae, or they may extend laterally, producing penetration plates (Hajek and Leger, 1994). The lateral growths may cause fractures that favor penetration (Brey et al. 1986) and may produce and disperse cuticle-degrading enzymes (Goettel et al. 1989).

Two distinct social forms of *S. invicta* exist: the monogyne or single queen colonies and polygyne or multiple queen colonies. Genetic and morphological evidence indicates that

both forms are of the same species (Ross and Fletcher 1985, Trager 1991); however, they differ in other aspects of their biology. Sex determination in the RIFA is haplodiploid; thus, unfertilized eggs become female workers and eggs fertilized with sperm stored in the spermatheca become male (thelytoky). The lack of recombination of the social B and social b (SB and Sb) chromosomes is responsible for the existence of two social forms of the RIFA (Wang et al. 2013). Monogyne (single queen) colonies are homozygous, possessing only the *General Protein-9* (Gp-9<sup>B</sup>) allele, whereas polygyne (multi queen) colonies are heterozygous and possess Gp-9<sup>B</sup> and Gp-9<sup>b</sup> alleles (Ross and Keller 1998). Queens of monogyne colonies (BB genotype) are heavier, more fecund, and longer lived compared to polygyne queens (Keller and Ross 1999, Gotzek and Ross 2007). The *Gp-9* gene codes for two groups of odor binding proteins and the allelic differences between social forms are correlated with behavioral differences (Krieger and Ross 2002). Workers from monogyne colonies are related to the queen by approximately 75%, whereas individuals from a polygyne colony possess varying degrees of relatedness. Monogyne workers aggressively defend their territory and kill foreign queens (Keller and Ross 1998). Due to low levels of relatedness, polygyne workers are less aggressive towards non-nestmate conspecifics (Obin et al. 1993, Vander Meer and Alonso 2002, Chirino et al. 2012) and readily exchange workers, brood, and food. This interaction between adjacent polygyne colonies could allow multiple nests to become interconnected into super colonies (Bhatkar and Vinson 1987). Polygyne supercolonies occur in very dense concentrations of 300-2,000 mounds per hectare (Lofgren and Williams 1984, Porter et al. 1988) compared to only 40-80 mounds per hectare for monogyne forms (Porter and Tschinkel 1987). Although both social forms are polymorphic,

monogyne colonies contain an increased proportion of larger workers (Greenberg et al. 1985). Workers from polygyne colonies are on average 16% smaller and it has been suggested that because the polygyne form has a higher proportion of smaller workers that polygyne fire ants can filter out smaller particles and are slightly less susceptible to infection in comparison to the monogyne form (Siebeneicher et al. 1992).

Strains of *Beauveria bassiana* and *Metarhizium spp.* utilized in our bioassays were originally isolated from native and exotic locales from both fire ants and alternate insect host species. The objective of the study described herein was to assess the pathogenicity and virulence of *Beauveria* and *Metarhizium* isolates collected from varying hosts in domestic and foreign locales against monogyne and polygyne *S. invicta* workers by evaluating the mycosis duration and mortality of ants directly exposed to pure, harvested conidia and a dose series of *B. bassiana* strain 447.

## **Materials and Methods**

*Solenopsis invicta* were collected from mounds located on roadsides throughout the state on North Carolina, USA. Locally, *S. invicta* were collected from mounds within one square mile of the NCSU Lake Wheeler Road Field Education Farm in Raleigh, NC. The number of mounds sampled varied from 1 to approximately 10 for each county. A minimum of 50 workers per mound were transported back to the laboratory within individual 9 dram plastic vials. Sample location was marked on a North Carolina state road map and a label placed within each vial. Collected ants were preserved in 70% EtOH and kept at -18°C until social form was assessed.

Definitive determination of monogyne or polygyne social form was conducted using PCR methods developed by Valles and Porter (2003). A DNA pellet was extracted by placing 20 to 30 ants from each field collected colony into a 1.7 ml microtube containing 142  $\mu$ l of lysis buffer (50mM Tris; pH8; 4% SDS) and ground with a pestle. Microtubes were subsequently incubated on a heat block at 100°C for 15 min prior to being placed on ice for 1 min. A total of 200  $\mu$ l of phenol:chloroform:isoamyl alcohol (25:24:1) was pipetted through the upper layer of the solution and the tube inverted 10 times. Following centrifugation at maximum speed for 5 min, 55  $\mu$ l of the relatively clear upper solution was removed and pipetted into 100  $\mu$ l of ice cold isopropanol which squeezed the DNA out of solution. Each microtube was subsequently flicked 3 times and centrifuged for 5 min. With a pellet formed at the bottom of the microtube, the liquid was slowly poured out and 0.5 ml of ice cold 70% EtOH added. The tube was centrifuged for 5 min and the EtOH slowly poured out which left the pellet at the bottom of the tube. The small amount of remaining solution was removed after an additional 20 sec of centrifugation. Tubes were incubated with tops open at 38°C for 5 to 10 min. Dependent upon the size of the DNA pellet, the pellet was resuspended in 50, 75, or 100  $\mu$ l of resuspension buffer (10mM Tris; pH8; 1mM EDTA) for 5 min and then placed on ice. At this point, samples were stored at -18°C until sufficient numbers of samples were available to be run through the thermocycler program.

In preparation for the thermocycler, 5  $\mu$ l of PCR buffer 10x, 2  $\mu$ l  $MgCl_2$ , 1  $\mu$ l dNTP, 0.4  $\mu$ l TAQ (2 units), 32.6  $\mu$ l DEPC treated  $H_2O$ , and 2  $\mu$ l of primers 16, 24, 25, and 26 (Integrated DNA Technologies, Coralville, IA) were mixed together per sample. Using a filtered pipette tip, 49  $\mu$ l of prepared solution was added to each microtube containing 1  $\mu$ l of

resuspended DNA and centrifuged for 5 sec. Samples were placed in the thermocycler and processed via a program of 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 15 sec/56°C for 15 sec/68°C for 45 sec, and 1 cycle of 68°C for 5 min.

A volume of 2.5 µl of loading buffer dye (methyl bromide) was placed onto Parafilm® and 15 µl of DNA sample was mixed into the buffer dye droplet using the tip of the pipette. The mixture was added to an individual well of a 1.8% agarose gel and the PCR gel electrophoresis ran for approximately 20 min at 140 volts. The molecular weight marker utilized had 8 bands ranging between 50 and 2000 base pairs and allowed the differentiation of monogyne and polygyne social form based upon the Gp-9 allele.

Fire ants used in bioassays were from laboratory maintained colonies that were initially selected for soil extraction based on the assessment of the number of mounds per unit area, average worker size, and the presence of a single or multiple queens within the colony. Social form was confirmed by PCR (Valles and Porter 2003). The ants used in laboratory bioassays were collected from Lake Wheeler Road in Raleigh, North Carolina via soil extraction and separated from soil and debris by way of a modified water drip technique that utilized a drip rate of 300 drops per minute (Chen and Wei 2005). Colonies were placed into plastic shoe boxes with the interior rim coated with a Fluon® water dilution of 1:25 (Chen 2007) and maintained at 25° C ± 3°C on a 12:12 light, dark cycle. A minimum of 15 monogyne and polygyne colonies were maintained in laboratory conditions for a maximum of one year prior to being replaced. An artificial nest cell was constructed from a Petri dish, painted black, and containing a thin layer of moistened plaster of Paris to maintain humidity as needed. Each colony was provisioned with 10-15 ml of a 10% sugar water mixture, a

diversified diet of orthopteran species collected by sweep net, darkling beetle, *Alphitobius diaperinus* (Panzer) larvae, and Vienna sausage three times a week. Ants utilized in experimental assays were taken from laboratory colonies containing 10,000 ants with visible brood and had a population index of 9 or 10 (Williams et al. 1999).

Strains of *B. bassiana* and *Metarhizium* were cultured on Sabouraud-maltose agar and supplemented with 1% yeast extract (SMAY). Two *B. bassiana* strains (BbP89, BbL90) were isolated from house flies, *Musca domestica* L., collected on a New York dairy in 1990 (Steinkraus et al. 1990); the third *B. bassiana* strain, (Bb447) collected by S. B. Alves in 1986 was originally isolated from *S. invicta* workers in the Mato Grosso of Brazil and acquired from the USDA-ARS Entomopathogenic Fungus collection ([www.ars.usda.gov/SP2UserFiles/Place/19070510/ALL%20AVAIL%2016Jan2014.pdf](http://www.ars.usda.gov/SP2UserFiles/Place/19070510/ALL%20AVAIL%2016Jan2014.pdf)). Two strains of *Metarhizium* isolated from fire ants were acquired from the USDA-ARS Collection of Entomopathogenic Fungus collection. One strain, *M. robertsii* (previously *M. anisopliae* (Metschnikoff) Sorokin) was isolated from fire ants in São Paulo, Brazil (Ma2561); a second strain, *M. brunneum* Petch (previously *M. anisopliae* (Metschnikoff) Sorokin) was originally isolated from *S. invicta* at College Station, Texas (Ma3738). A third strain of *Metarhizium* (MaNC) was isolated from an American cockroach, *Periplaneta americana*, collected in North Carolina (Zurek et al. 2002). Dry conidia were added to flasks containing liquid culture composed of 150ml of cooled sterile Bacto™ Sabouraud Maltose Broth supplemented with 0.15g yeast extract (Anonymous 1984) and mycelia were allowed to grow on a 100 rpm rotary shaker at approximately 23°C for 5-10d. Subsequently, conidia were mass produced using a modified production method described by Alves and Pereira

(1989). The liquid culture was injected into autoclaved bags of hydrated white rice. After 14d of growth within a 25°C incubator, the autoclaved bags were opened to promote drying for 7-14d. Conidia were separated from the dried rice and fungus mixture via a 100-mesh sieve with an opening of 0.120mm. Fungi were reisolated from a second passage through ants, grown on rice, and preserved at -80°C until used in bioassays. Conidial concentrations per mg of dry material were calculated using a hemocytometer as  $2.2 \times 10^7$ ,  $3.4 \times 10^7$ ,  $1.7 \times 10^7$  for BbP89, BbL90, and Bb447, respectively; the *Metarhizium* strains Ma2561, Ma3738, and MaNC had conidial concentrations of  $7.6 \times 10^6$ ,  $9.1 \times 10^6$ , and  $1.1 \times 10^7$  conidia per mg of dry material, respectively.

Laboratory assays were initiated by transferring cohorts of approximately 50 monogyne and polygyne fire ants from rearing trays to 9 dram plastic vials containing approximately 1 gram of conidia. Workers from a single colony were used to assess each strain of entomopathogen. Initial applications in order to evaluate the efficacy of *Beauveria* and *Metarhizium* conidial treatments against *S. invicta* were undiluted and replicated four times. Negative controls were inserted into vials containing bleached white flour only. Capped vials were slowly agitated with ants exposed to the dry contents for 1 minute. Vials were opened, positioned at an incline over the upturned cap within the experimental arena, and all ants were permitted to climb out of the vial and into arenas provisioned with sugar water and nest cells.

Dose-response experiments were conducted with 3 dosages of Bb447 conidia and replicated three times. The Bb447 strain was selected for the dose-response bioassay because all three *B. bassiana* strains provided equivalent control when conidia were undiluted and

Bb447 has been historically utilized to assess efficacy against *S. invicta* in both laboratory and field conditions. Monogyne and polygyne ants were exposed to 0.01 g of conidia mixed into a 1:100, 1:50, or 1:10 dilution of bleached wheat flour. Weights of the dry material that remained in the plastic vial after all ants had exited were recorded and the average number of conidia exposed to an individual ant estimated. A uniform mixture of conidia within the flour was assumed. The estimated number of conidia per mg was divided by 100 and the total multiplied by the percentage of dry material on each ant cohort. The average number of conidia per ant was estimated after tests had been initiated with an average inoculum dose per monogyne ant calculated to be  $1.22 \times 10^5$ ,  $2.44 \times 10^5$ ,  $4.95 \times 10^5$ ; polygyne ants were exposed to an average of  $9.62 \times 10^4$ ,  $1.28 \times 10^5$ ,  $3.54 \times 10^5$  conidia.

Experimental arenas were identical to the rearing trays used to maintain laboratory colonies. Cohorts were maintained in ambient laboratory conditions ( $\approx 20$  - $25^\circ\text{C}$ ). Dead ants were collected daily for 9 d, counted, and placed on moistened filter paper to provide conditions for sporulation of fungus-infected individuals. Cadavers with emerging conidia were considered positive for fungal infection and visually confirmed via dissecting microscope. At the end of each experiment, cumulative mortality was calculated by dividing the total number of infected ants within each experimental cohort by the sum of cadavers and survivors. Corrected mortality was calculated according to Henderson and Tilton (1955), arcsin square root transformed in order to meet normality and variance assumptions, and compared by analysis of variance (ANOVA) procedure ( $P = 0.05$ ) (Minitab Statistical Software v 16, Minitab Inc, PA, USA). The pathogenicity of fungal isolates were analyzed using a general linear model in which isolate and social form were treated as independent

variables and the arcsin square root proportion of dead ants was treated as the response variable.

## Results

### *Survey of social forms in NC counties*

*Solenopsis invicta* were collected from mounds located on roadsides in 30 of 100 counties within the state on North Carolina (Fig. 1). The large and small circles within the 30 counties of North Carolina signify where *S. invicta* were collected. Large circles correlate to counties in which a minimum of 5 mounds were sampled; small circles are associated with counties in which less than 5 mounds were observed or sampled. Numbers within the larger circles correspond to the percentage of polygyne mounds positively identified in that county via PCR. The color hue of the circle represents 5 ranges of overall percent polygyne form detected in the county. The map in Fig. 1 represents the counties that are under quarantine in 2010 and does not represent the true distribution of fire ants in North Carolina. The monogyne social form was predominantly sampled in the northeast; conversely, the central and northwestern edges of the quarantine map had a majority of *S. invicta* being of the polygyne social form.

### *Efficacy of undiluted Beauveria and Metarhizium isolates*

All strains tested were able to infect *S. invicta* in the laboratory (Fig. 2). Higher total mortality was observed with *Beauveria* strains than *Metarhizium* strains ( $F = 511.39$ ,  $df = 47$ ,  $P \leq 0.001$ ). Irrespective of fire ant social form, all *Beauveria* strains provided 100%

mortality 6 d after exposure to undiluted conidial concentrations. Average mortality was influenced by *Metarhizium* strain with reduced mortality to ants exposed to the MaNC strain isolated from a cockroach (Table 1); however, overall susceptibility to infection was not impacted by social form ( $F = 0.04$ ,  $df = 23$ ,  $P = 0.837$ ).

#### *Efficacy of Bb447 at various doses*

At 3 d post-exposure, experiments in which Bb447 conidia were diluted with different amounts of bleached wheat flour and applied directly on the ants showed an average mortality rate of  $7.96 \pm 5.06\%$  and was similar to cohorts of monogyne ( $10.42 \pm 5.59\%$ ) and polygyne ( $11.14 \pm 7.23\%$ ) ants exposed to undiluted Bb447 conidia. Mortality of ants increased dramatically on the fourth day after application (Table 2). At 4 d after initial exposure, cumulative mortality rates differed by the average dose of conidia per ant ( $F = 55.82$ ,  $df = 2$ ,  $P \leq 0.001$ ). Cumulative monogyne mortality (mean  $\pm$  SEM) after 9 d was  $5.61 \pm 1.00\%$  for the control compared with  $87.97 \pm 1.56$ ,  $93.69 \pm 2.77$ , and  $95.79 \pm 1.82\%$  for increasing doses of conidia per ant. Cumulative polygyne mortality (mean  $\pm$  SEM) after 9 d was  $3.46 \pm 1.26\%$  for the control compared with  $88.60 \pm 1.56$ ,  $93.64 \pm 2.00$ , and  $93.34 \pm 1.10\%$  for increasing doses of conidia per ant.

A dose-response was observed for strain Bb447 (Table 2). A statistically lower survival rate at 4 d after exposure was detected in both social forms exposed to the low dilution. The middle dose of Bb447 exposed to monogyne ants ( $1.22 \times 10^5$ ) was most similar to the lowest dose exposed to polygyne ants ( $1.28 \times 10^5$ ). At a dose of approximately  $1 \times 10^5$  conidia per ant, polygyne ants had statistically lower survival compared to monogyne

ants. After 9 d, no statistical difference in cumulative mortality was detected between social forms or conidial dose per ant ( $F = 1.38$ ,  $df = 5$ ,  $P = 0.299$ ).

## Discussion

*Solenopsis invicta* was first identified in the southeastern quadrant of North Carolina in Brunswick County in 1957 ([www.ncagr.gov/plantindustry/plant/entomology/IFA.htm](http://www.ncagr.gov/plantindustry/plant/entomology/IFA.htm)). As evidenced by survey results, monogyne and polygyne social forms are wide spread in North Carolina, with polygyne forms being most prominent along the edges of the quarantine zone. Human transportation of the polygyne social form to the leading edge of the quarantine zone is likely because physical limitations of natural colony expansion is primarily through budding (Vargo and Porter 1989, King et al. 2009). This phenomenon has also been recently detected in the recently invaded country of China where the polygyne social form of *S. invicta* is detected most frequently at outlier sites (Yang et al. 2012).

In our study, monogyne and polygyne *S. invicta* were susceptible to all exotic and native isolates of *Beauveria* and *Metarhizium*, but mortality rates were significantly higher in ants exposed to all of the *Beauveria* isolates. There are examples of fungal isolates from a distinct host insect being highly virulent to an alternate target pest (Feng and Johnson 1990, Shan and Feng 2010). The two *Beauveria* strains that were originally isolated from house flies collected on a New York dairy (BbP89, BbL90) were highly virulent to *S. invicta* and this is the first time reported to be efficacious to *S. invicta*. The BbL90 isolate provided an average mortality increase of 22.65% (monogyne) and 12.38% (polygyne) to fire ants after 4 d compared to the Bb447 strain. All three strains of *Beauveria* tested provided 100%

mortality by 6 d after exposure to undiluted conidia and our results conform to observations by Madelin (1963) that mortality due to *B. bassiana* is variable, often ranging from 3 to 7 days.

*Beauveria* and *Metarhizium* are ubiquitous entomopathogens that are prevalent in a large number and diverse group of arthropods (Veen 1968, Goettel et al. 1990). Most fungal isolates have a restricted host range (Goettel et al. 1990, Vestergaard et al. 2003, Zimmermann 2007a); however, the host range of *B. bassiana* is less restricted than that of *M. anisopliae* (Zimmermann 2007b). Higher mortality rates observed in this study and in *Camponotus pennsylvanicus* workers (Kelley-Tunis et al. 1995) may reflect a broader host range for *B. bassiana* than *M. anisopliae*.

Typically, virulence of differing strains of *Metarhizium* (Ferron et al. 1972, Rombach et al. 1986, Bidochka and Small 2005, Zimmermann 2007b) and *Beauveria* (Goettel et al. 1990, Feng et al. 1994, Vestergaard et al. 2003, Zimmermann 2007a) is highest in the original host species. We observed the lowest average mortality ( $33.12 \pm 4.13\%$ ) to ants exposed to the *Metarhizium* strain originally isolated from a cockroach cadaver collected in North Carolina; the reduced efficacy in our test supports the notion that MaNC virulence to fire ants may be limited because the strain was originally isolated from a blattid.

*Metarhizium* strains isolated from Brazil (Ma2561) and Texas (Ma3738) had conidial concentrations of  $7.6 \times 10^6$  and  $9.1 \times 10^6$ , respectively, and were less virulent than observed in prior studies. In our laboratory bioassays, Ma2561 and Ma3728 provided an average of  $63.95 \pm 3.49\%$  control after 9 d of exposure. *Metarhizium anisopliae* has been previously reported to provide 100% mortality to *S. invicta* queens after five days when evaluated in

controlled conditions (Sanchez-Peña 1992). Decreased virulence and lower susceptibility levels associated with ants exposed to *Metarhizium* may be attributed to differences in production methodologies that ultimately impacted conidial concentrations (Steinhaus 1949, Feng et al. 1994, Lopes and Alves 2011). The study was terminated at 9 d after exposure which was 3 d after all ants exposed to the three *Beauveria* strains had died. The time interval between conidia exposure to last observation of mortality may have not been long enough to evaluate complete efficacy of the *Metarhizium* strains.

Fire ants that make direct contact with spores or ingest them are invaded and killed (Broome et al. 1976). Conidia germinating on the tarsi and within buccal pellets of fire ants suggest that the head and legs are the major routes of infection (Siebeneicher et al. 1992); however, due to the heavy sclerotization of *S. invicta*, Charnley (1989) notes that fungal pathogens often invade workers via spiracles. Recent scanning electron microscopy observations on the infection process of *B. bassiana* conidia on the cuticle of *S. invicta* illustrated that most conidia germinate and the fungus penetrate the host at intersegmental membranes, setae, and tibia between 18 h to 24 h after exposure (Wang et al. 2010). Complete ingestion of inocula is often prevented because the conidia are collected in the infrabuccal pocket on the ventral surface of the buccal cavity and expelled as a pellet (Eisner and Happ 1962; Siebeneicher et al. 1992). On average, *S. invicta* workers filter particles larger than 0.864 microns from their food but filtration is influenced by ant size (Glancey et al. 1981). Siebeneicher et al. (1992) observed major workers of *S. invicta* are capable of ingesting *B. bassiana* conidia with germinating conidia present in the crop upon dissection; thus, susceptibility to pathogens is influenced by the size of an individual worker (Kermarrec

et al. 1990, Müller et al. 1996, Naug and Camazine 2002). Monogyne colonies contain a higher proportion of larger workers (Greenberg et al. 1985); because of this divergence between social forms, it has been suggested that the smaller polygyne form can filter smaller particles and is slightly less susceptible to infection in comparison to the monogyne form (Siebeneicher et al. 1992). In our test, no statistical difference in cumulative mortality was detected between social forms at 9 d post-exposure.

Ant management tactics currently include contact insecticides applied either as a barrier (Rust et al. 1996, Wiltz et al. 2010), broadcast (Drees et al. 2000), or mound treatments (Williams and Lofgren 1983); the use of baits (Glancey et al. 1973, Banks 1986) has increased target specificity with reduced toxicant volumes (Klotz et al. 1997).

Homeowner perceptions and concerns over the usage of synthetic pesticides deleteriously impacting environmental contamination coupled with the notion that homemade treatment remedies are efficacious has amplified the interest and popularity in the usage of less toxic or “natural” substances for insect control (Drees and Lennon 1998, Potter and Bessin 1998, Baldwin et al. 2008). It was shown that both native and exotic strains of naturally occurring *B. bassiana* are able to provide complete control *S. invicta* in laboratory conditions and have the potential to be used as biocontrol agents against *S. invicta*. In the United States, management of fire ants with the entomopathogen *B. bassiana* has historically assessed the Bb447 strain. Control provided by the BbP89 and BbL90 strains warrant further investigation in field conditions for use in an IPM program.

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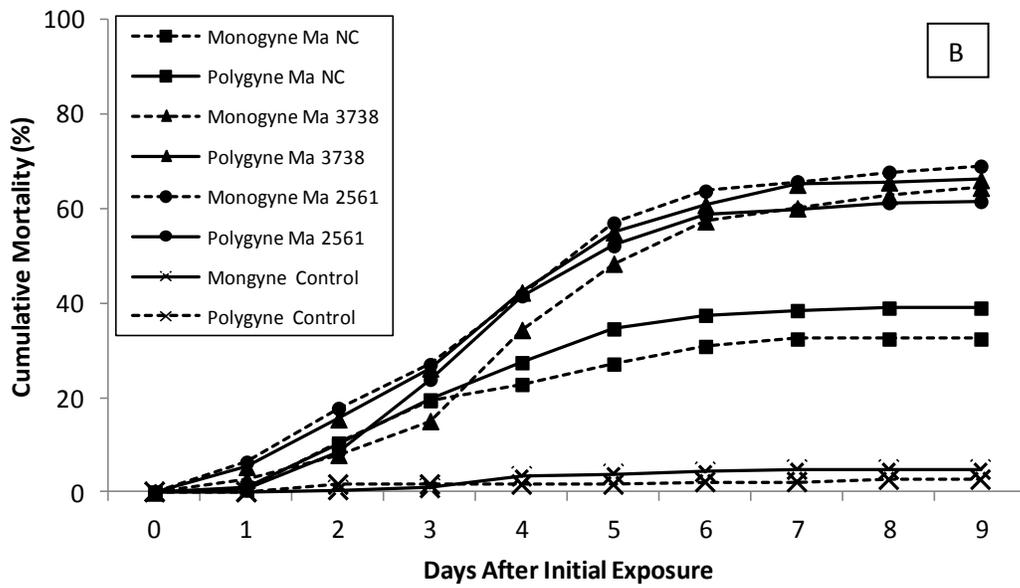
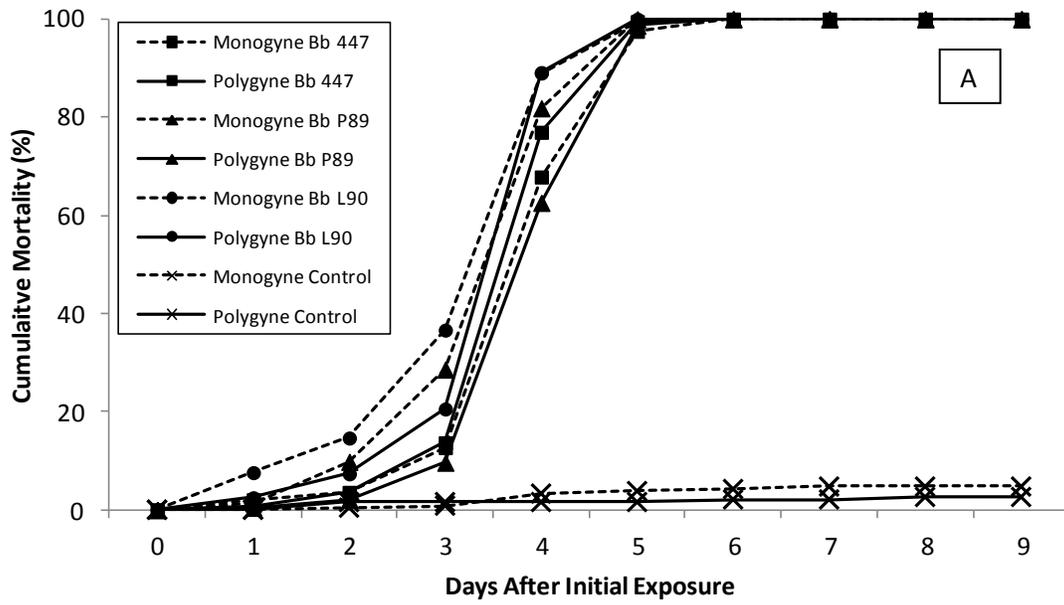
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**Figure 2** Cumulative percent mortality of monogyne and polygyne red imported fire ant cohorts exposed to undiluted strains of *Beauveria* (A) and *Metarhizium* (B) and maintained in trays in the laboratory. *Beauveria* dosages correspond to  $2.2 \times 10^7$ ,  $3.4 \times 10^7$ ,  $1.7 \times 10^7$  for BbP89, BbL90, and Bb447, respectively; and *Metarhizium*  $7.6 \times 10^6$ ,  $9.1 \times 10^6$ , and  $1.1 \times 10^7$  conidia per mg of dry material, for Ma2561, Ma3738, and MaNC, respectively.



**Table 1** Mean mortality (Means  $\pm$  SE) of differing social forms of *S. invicta* at 2, 3, 4, 5, and 9 d after exposure to three strains of *Beauveria* and *Metarhizium*

Social Form	Strain	Means $\pm$ SE % mortality on day				
		2	3	4	5	9
Monogyne	Bb 447	3.14 $\pm$ (0.94)cd	11.93 $\pm$ (2.59)de	67.12 $\pm$ (7.34)b	97.48 $\pm$ (1.94)a	100.00 $\pm$ (0.00)a
	Bb P89	9.43 $\pm$ (1.86)abc	28.00 $\pm$ (2.84)ab	81.49 $\pm$ (5.78)ab	100.00 $\pm$ (0.00)a	100.00 $\pm$ (0.00)a
	Bb L90	14.25 $\pm$ (5.13)ab	36.06 $\pm$ (3.74)a	89.77 $\pm$ (2.39)a	99.48 $\pm$ (0.52)a	100.00 $\pm$ (0.00)a
	Ma1	10.02 $\pm$ (1.08)abc	18.69 $\pm$ (1.14)bcde	20.05 $\pm$ (3.73)d	24.05 $\pm$ (5.22)d	28.99 $\pm$ (3.32)c
	Ma2	7.51 $\pm$ (0.67)abc	14.36 $\pm$ (2.52)cde	32.09 $\pm$ (2.86)d	46.23 $\pm$ (2.50)bc	62.67 $\pm$ (1.67)b
	Ma3	17.37 $\pm$ (1.57)a	26.33 $\pm$ (2.39)abc	39.62 $\pm$ (3.79)cd	55.15 $\pm$ (3.16)b	67.44 $\pm$ (2.27)b
Polygyne	Bb 447	2.59 $\pm$ (1.50)cd	12.21 $\pm$ (3.50)de	76.50 $\pm$ (6.30)ab	99.49 $\pm$ (0.51)a	100.00 $\pm$ (0.00)a
	Bb P89	1.61 $\pm$ (2.01)d	8.12 $\pm$ (3.10)e	61.97 $\pm$ (3.00)bc	98.66 $\pm$ (1.34)a	100.00 $\pm$ (0.00)a
	Bb L90	5.88 $\pm$ (2.17)bc	19.18 $\pm$ (4.35)bcd	88.88 $\pm$ (2.33)a	100.00 $\pm$ (0.00)a	100.00 $\pm$ (0.00)a
	Ma1	8.71 $\pm$ (0.68)abc	18.24 $\pm$ (1.38)bcde	26.22 $\pm$ (2.37)d	33.50 $\pm$ (1.43)cd	37.25 $\pm$ (2.23)c
	Ma2	14.05 $\pm$ (1.48)ab	24.99 $\pm$ (2.45)abc	41.27 $\pm$ (3.45)cd	54.25 $\pm$ (2.47)b	65.16 $\pm$ (1.84)b
	Ma3	7.15 $\pm$ (0.97)abc	22.71 $\pm$ (2.23)abcd	40.62 $\pm$ (0.95)cd	51.39 $\pm$ (1.77)b	60.46 $\pm$ (2.41)b

Note. Means presented in this table were corrected for control mortality (Henderson and Tilton 1955) from each test day. Mean control mortality for monogyne and polygyne ants were 4.72%  $\pm$  1.21 and 2.60  $\pm$  1.35, respectively. Means associated with the same letter in a column are not significantly different Tukey; 95% CI.

**Table 2** Mortality of monogyne and polygyne *S. invicta* exposed to three dilutions of *Beauveria bassiana* (Bb447)

Social Form	Weights (g)		Average conidia per ant	Means $\pm$ SE % mortality by day after exposure	
	Conidia	Flour		% Mortality at 4 d	% Mortality at 9 d
Monogyne	0.01	0.1	$4.95 \times 10^5$	52.83 ( $\pm$ 2.68) a	95.79 ( $\pm$ 1.82) a
Monogyne	0.01	0.5	$2.44 \times 10^5$	42.25 ( $\pm$ 1.54) ab	93.69 ( $\pm$ 2.77) a
Monogyne	0.01	1.0	$1.22 \times 10^5$	27.04 ( $\pm$ 1.16) c	87.97 ( $\pm$ 1.56) a
Polygyne	0.01	0.1	$3.54 \times 10^5$	49.60 ( $\pm$ 3.69) a	93.34 ( $\pm$ 1.10) a
Polygyne	0.01	0.5	$1.38 \times 10^5$	49.56 ( $\pm$ 2.38) a	93.64 ( $\pm$ 2.00) a
Polygyne	0.01	1.0	$9.62 \times 10^4$	36.40 ( $\pm$ 1.83) bc	88.60 ( $\pm$ 1.56) a

Note. Means presented in this table were corrected for control mortality (Henderson and Tilton 1955) from each test day. Mean control mortality for monogyne and polygyne ants were  $5.61\% \pm 1.00$  and  $2.60 \pm 1.35$ , respectively. Means associated with the same letters in a column are not significantly different; Tukey; 95% CI.

## CHAPTER 2

Caldwell: Behavioral changes of *Solenopsis*  
*invicta* exposed to *Beauveria bassiana*

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**Prevalence and implications of *Solenopsis invicta* (Hymenoptera: Formicidae) altered behavior after exposure to *Beauveria bassiana* (Hypocreales: Clavicipitaceae)**

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**Abstract.** Behavioral changes can occur to insects in response to an infection. Summit disease is described as altered normal host behavior with vertical movement hypothesized to maximize the dissemination efficacy of a pathogen; conversely, a behavioral fever response is hypothesized to be an attempt by the infected host to thermoregulate in order to eliminate the pathogen by moving to an area with higher ambient temperatures which is lethal for the entomopathogen but not the host. Whether these behavioral changes are mechanisms of host defense, manipulations by the parasite or coincidental events is not always clear. Red imported fire ants, *Solenopsis invicta*, infected with *Beauveria bassiana* (Bb447) was evaluated for behavioral changes associated with phototactic responses, elevation seeking, and movements over a heat gradient. Infected ants were not attracted to lighted conditions with an average of  $\geq 67\%$  of conidia exposed ants remaining in the dark portion of a laboratory choice arena. A mark and recapture bioassay showed that climbing events began on day 2 and peaked on day 4 with 9.3% of all ants located on vertical surfaces. Average time between original collection from a vertical surface, marked with paint, and the cadaver recovered from the arena was 1.32 d; 93% of ants were previously unmarked. After 6 h within the heat gradient, most control ants ( $86 \pm 13\%$ ) were aggregated within the  $< 27^\circ\text{C}$  zone and remained there for the entirety of the 5 day assay. An average of 47% and 31% of the treated ants were located at temperatures  $> 27^\circ\text{C}$  on day 2 and 3, respectively. Observations associated with the timing of increased movement by infected workers confirm prior laboratory experiments. Based on lack of survival in the heat gradient choice assay and the dispersion and movement upwards during late infection period, it is suggested that the

alteration in normal host behavior is a benefit to Bb447 and increases opportunities for dissemination enhancement.

**Keywords.** Elevation seeking, summit disease, phototaxis, temperature, fire ant, *Solenopsis*, *Beauveria*

## Introduction

Interactions between entomopathogenic fungi and a susceptible host involve a complex array of biological and behavioral modifications to which the microbial pathogens are often reliant upon for spore proliferation and dispersal. Parasitic manipulation of a host's phenotype can alter the behavior of the host they inhabit with resulting transmission enhancement at the expense of host fitness (Moore and Freehling 2002, Hughes 2014, Weinersmith and Faulkes 2014). Summit disease is described as the vertical movement of an infected host to higher elevations and is theorized to maximize the dissemination efficacy of a pathogen by altering normal host behavior in order to disperse infective propagules with greater conduciveness (Marikovsky 1962, Roy et al. 2006). Boer (2008) observed wood ants, *Formica rufa*, exhibiting summitting disease symptoms with infected ants adhered to vegetation above and adjacent to mounds and were optimal positions for the host to sporulate and reinfest nestmates. However, poikilothermic hosts are known to initiate a defensive febrile response to ward off lethal infection (Louis et al. 1986, Boorstein and Ewald 1987, Kluger 1991, Watson et al. 1993). A behavioral fever response is hypothesized to be an attempt by the infected host to thermoregulate in order to eliminate the pathogen by moving to an area with higher ambient temperatures which is lethal for the entomopathogen but not the host (Marikovsky 1962, Hutchison and Erskine 1981, Roy et al., 2006). Infection with bacteria, fungi, microsporidia, and macroparasites (including tachinids), has elicited a behavioral fever response documented in multiple species in several different orders (Fisher and Hajek 2014). Elevation seeking by insects at late stages of viral and fungal infection has been observed in a diversified group of species within the orders Lepidoptera (Hoffman

1891, Steinhaus 1949, Steinkraus et al. 1993) Orthoptera (Skaife 1925), Coleoptera (Roy et al. 2006) Hemiptera (Harper 1958), Diptera (Roy et al. 2006) and Hymenoptera (Zimmermann 2007). The description of altered behaviors associated with elevation seeking, observed days after exposure to a pathogen, may be related to a fever response or summit disease syndrome.

Isolated from *S. invicta* workers in Brazil, the pathogenicity of the soil fungus *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin has been described (Stimac et al. 1987, Alves et al. 1988, Pereira et al. 1993, Oi et al. 1994) and has decreased populations in laboratory and field assays with varying and inconsistent success. Inconsistent efficacy can be attributed to normal host immune responses, soil moisture and composition (Fuxa and Richter 2004), and ant behaviors such as antimicrobial secretions from the venom sac and metapleural glands (Storey et al. 1991), grooming, nest hygiene, avoidance behaviors, dispersal, and colony movement (Oi and Pereira 1993). The most frequently observed behavioral response of ants infected with fungal pathogens is movement away from the colony (Oi and Pereira 1993). Pereira and Stimac (1992) conducted laboratory experiments involving small *Solenopsis invicta* colonies infected with *B. bassiana*; subsequently, infected ants were observed wandering outside of the nest three to five days, post-inoculation. In field injections of *B. bassiana* into *S. invicta* colonies, behavioral alterations associated with erratic movements to the tops of the blades of grass were observed (Oi et al. 1994).

Parasitic infection often induces behavioral alterations in the host which may ultimately benefit the host or the parasite; however, whether these behavioral changes are

mechanisms of host defense, manipulations by the parasite or coincidental events is not always clear. Although the vertical movement of infected individuals has been observed and induced in field studies of *S. invicta* (Oi et al. 1994), the elevation seeking behavior has not been experimentally induced and demonstrated quantitatively in laboratory conditions. An objective of this research was to evaluate the altered behavior of infected fire ants exposed to *B. bassiana* (447) and determine the prevalence of the elevation seeking behavior of *S. invicta* exposed to *B. bassiana* (447) in order to determine if the frequency and timing of summitting events is consistent and predictable or if the observed climbing behavior of infected ants is merely a function of conspicuousness and is thus over reported as a common phenomenon. Secondly, studies were designed to determine the levels of patent infection and eventual mortality to *B. bassiana*-infected *S. invicta* workers when provided the opportunity to thermoregulate on a heat gradient.

### **Materials and Methods**

Colonies of *S. invicta* were collected in North Carolina, social form confirmed via PCR, and maintained in laboratory conditions (Chapter 1). The Bb447 strain of *B. bassiana* was originally isolated from *S. invicta* workers in Brazil, registered for use in the United States in 2002, and utilized in all experimental bioassays. The Bb447 strain was obtained from the USDA-ARS Collection of Entomopathogenic Fungus Cultures. Pure cultures were grown on sterile rice, and preserved at -80°C until used in bioassays (Chapter 1). A conidial concentration of  $2.9 \times 10^9$ /mg of dry material was calculated using a hemocytometer.

All laboratory assays were initiated by placing cohorts of 50 fire ants from the same colony into individual 9 dram vials containing flour (0.01g) and conidia (0.001g). Vials containing the dry contents were stirred with a sterile loop and vortexed. Ratios of flour to conidia were selected to extend the infection period and approximated an LD<sub>90</sub>. Negative controls were included in all tests with workers being taken from the same field-collected colony as the conidia exposed ants. Ants of the control replicates were inserted into vials containing only flour but were otherwise handled and observed in the same manner as the conidia exposed ants. Ants within the capped vials were slowly agitated and remained for one minute. Each vial was positioned at an incline over the upturned cap and all ants were permitted to climb out of the vial and into the center of the arena. All experimental arenas were provisioned with sugar water and an artificial nest. Experimental arenas were identical to the plastic boxes used to maintain the laboratory colonies. Doses were calibrated by taking pre and post-weights of the dry contents and the percentage of material on the ants calculated. Uniformity of conidia mixed throughout the flour was assumed. The estimated number of conidia per mg ( $2.9 \times 10^9$ ) was divided by 100 and the total multiplied by the percentage of dry material on the ants ( $n = 50$ ). The average number of conidia per ant was determined by dividing the estimated number of conidia per ant cohort by 50 and equated to  $2 \times 10^6$ - $10^7$  conidia per ant. Cadavers were removed daily and placed on a moistened filter paper within a sealed Petri dish in order to promote sporulation, indicative of patent infection, two to three days later (Steinkraus et al 1990).

At the conclusion of each experiment, cumulative mortality was calculated by dividing the number of cadavers with emerging conidia by 50. Corrected mortality was

calculated with the Abbott's formula (Abbott 1925). Percent mortality data was arcsin square root transformed in order to meet normality and variance assumptions and compared by analysis of variance (ANOVA) procedure ( $P = 0.05$ ) (Minitab Statistical Software v 16, Minitab Inc, PA, USA).

### *Experiment 1: phototaxis*

Half of each experimental arena had the exterior portion of the walls and floor painted black. A black painted lid was cut in half and positioned over the dark portion. A fabricated plastic flap was secured with tape between the lid, floor, and walls of the arena. The two access points between the light and dark portions of the arena were limited to the floor edges and measured 1 cm by 1 cm. Each side of the arena was provisioned with an artificial nest and sugar water. The artificial nest within the dark portion of the arena was painted black; whereas, the light portion remained unpainted. The vials containing conidia exposed or control ants were positioned at the midline between the light and dark portions of the arena. The number of ants observed in the light portion of each arena was recorded at 1000 h and 1800 h, through day six. Determination of the number of ants within the dark portion of the arena was calculated by subtracting the number of ants counted in the light portion from 50. The experiment was replicated three times with initiation dates one day apart. Phototactic response after exposure to conidia between 0 d and 6 d post-exposure were analyzed using a general linear model in which exposed and unexposed ants, day, and time were treated as independent variables and the proportion of ants in the lighted portion of the arena was treated as the response variable.

### *Experiment 2: elevation seeking*

A 183 cm bamboo stake was inserted into a predrilled, 2.54 cm diameter PVC end cap and glued to the central floor of each arena. String was connected from the four corners of the arena to a point on the bamboo stake measuring 25 cm above the floor and provided stability to the bamboo stake and increased the opportunity of ants being located on the stake during observations. Two separate assays were conducted. The first assay compared climbing behavior of monogyne and polygyne social forms of *S. invicta* exposed to Bb447 conidia. Location of climbing ants were recorded based upon zone numbers with all individuals located on the arena wall, strings, and the first 30.5 cm of the bamboo stake recorded as being in zone 1. Zones 2 through 6 were incremental increases of 30.5 cm. Heights and locations of ants were recorded daily at 0900 h and 1700 h, between zero and five days post-exposure. Each replicate ( $n = 3$ ) consisted of 50 ants. Both social forms were evaluated. Comparison of average climbing height of ants for both monogyne and polygyne social forms was compared by analysis of variance (ANOVA) procedure ( $P = 0.05$ ) by day after exposure (Minitab Statistical Software v 16, Minitab Inc, PA, USA); social form data was pooled and compared to the untreated replicates.

The second climbing assay utilized mark and recapture technique and evaluated the timing of increased movement by conidia exposed monogyne ants and whether climbing events were performed by new, unmarked ants. During each observation time period, ants climbing on all vertical surfaces were collected and marked with Testors™ paint (Bhatkar et al. 1991) and returned to their respective arenas while the paint was still wet (Wojcik et al. 2000). All summing ants on day 2 and 2.5 were marked on the head (mark 1), on day 3 and

3.5 ants were marked on the thorax (mark 2), on day 4 and 4.5 ants were marked on the upper abdomen / petiole (mark 3), and on day 5 and 5.5 ants were marked on the lower abdomen (mark 4). Dead ants at 2.5 days and beyond were evaluated via dissecting scope for the presence and location of paint. Ants located on vertical surfaces were collected, evaluated for previous markings, painted, and returned to their respective arena. Mark and recapture assays utilized approximately 50 ants per replicate (n=6) with observations conducted two times per day for 7 days.

### *Experiment 3: behavioral fever response*

Four aluminum baking pans (5.0 by 24.5 by 34.0 cm), with the edges coated with Fluon®, were utilized as experimental arenas in a thermoregulation choice assay in order to assess the movement and location of *B. bassiana* 447 exposed polygyne *S. invicta* workers. A stainless steel sheet was positioned directly underneath each aluminum pan and eliminated temperature fluctuations on the edges of the aluminum pans. Wingnuts and bolts were attached to the ends of each pan and allowed for alterations in the spacing between the aluminum pan and stainless steel plate. Underneath the stainless steel plates, two flexible strips of heat insulation ceramic fiber tape were positioned horizontally across the narrow portions of each pan. One fiber tape strip was located at the upper end of the pan and calibrated to provide a surface temperature of 48°C; the second strip was located at the midway point of the pan and provided a surface temperature of 34°C. The lower edge of each aluminum pan extended off of the workbench with a surface temperature equal to the room temperature of  $22.5 \pm 1^\circ\text{C}$ . Seven temperature zones were separated equidistantly

across the pan and were as follows: zone 1 (<27°C), zone 2 (27-30°C), zone 3 (30-33°C), zone 4 (33-36°C), zone 5 (36-39°C), zone 6 (39-42°C), zone 7 (>42°C). The temperatures of zones 3 thru 7 were recorded from thermal couples glued directly to the stainless steel sheet; however, the temperature on the surface of the aluminum pan was lower due to heat displacement and was calibrated accordingly. Sugar water was available within each temperature zone. Ant locations were recorded every two hours from the time of introduction into the arena (day 0), throughout the latent infection period (day 5), between the hours of 0800 to 2000. Comparison of the percentage of untreated and conidia exposed *S. invicta* located in temperature zones above 27°C was data was compared by analysis of variance (ANOVA) procedure ( $P = 0.05$ ) by time and day after exposure (Minitab Statistical Software v 16, Minitab Inc, PA, USA).

## Results

### *Experiment 1: phototaxis*

There was no increase in the proportion of ants exposed to a mixture of Bb447 conidia and flour being attracted to the lighted portion of the experimental arena throughout the six day experiment ( $F = 0.98$ ,  $df = 83$ ,  $P = 0.510$ ) (Fig. 1). An average of 67% or more of the conidia exposed ants remained in the dark portion of the arena on all days with no statistical difference to the untreated controls ( $F = 0.55$ ,  $df = 6$ ,  $P = 0.765$ ). In the light portion of arenas, conidia exposed ants were observed actively transporting cadavers or exhibiting altered behaviors such as wandering or remaining stationary on the walls of the arena at two days after assay initiation and beyond; conversely, untreated ants were

aggregated within the artificial nest or at the sugar water station. The assay ended on day six because all conidia exposed arenas contained 8 or fewer surviving ants from the original 50 (Table 1). All conidia exposed cadavers were confirmed to have been infected with *Beauveria*.

### *Experiment 2: elevation seeking*

Both social forms of *S. invicta* exposed to Bb447 followed a similar trend for height by day (Fig. 2). Average climbing heights increased daily until day 3 with average peak heights of 80 cm for monogyne and polygyne collectively (Fig. 3). No significant differences associated with average height were detected between social forms at day 3 ( $F = 0.09$ ,  $df = 1$ ,  $P = 0.782$ ) or any other observation time. Average mortality (mean  $\pm$  SEM) at three days post-exposure was  $31.7 \pm 3.2\%$  and  $24.0 \pm 3.5\%$  for the monogyne and polygyne social forms, respectively (Fig. 4); twenty-four hours later, mortality to both social forms had increased by 43%. The decrease in climbing events and average heights decreased at 3.5 days and beyond and correlated to the increase in accumulative mortality. No summing ants were observed on the fifth day.

In the mark and recapture experiment, the majority of conidia exposed ants that were collected from vertical surfaces were not marked with paint. Of the 129 ants collected, marked, and returned to their respective arenas between days 2 and 5.5, 93% of ants were previously unmarked (Fig. 5). Climbing observations peaked on the morning of day 4 with 9.3% of all ants initially exposed to Bb447 located on vertical surfaces; 53.8% of ants marked on day 4 were dead at day 4.5. The average time between an ant being initially

marked and the cadaver removed from the experimental arena was 1.32 days. After seven days, an average of 94.8% of all conidia exposed ants had died. A total of 275 cadavers were collectively removed over the entirety of the assay of which 24.73% of ants were marked. Six treated ants were recorded to have climbed a vertical surface on consecutive days with paint located on multiple body regions. Thirteen control ants were recorded on vertical surfaces over 7 days, painted, and returned to the experimental arena. No control ants were marked twice and no painted control ants died.

### *Experiment 3: behavioral fever response*

After 6 h within the gradient, most control ants ( $86 \pm 13\%$ ) were aggregated within the  $<27^{\circ}\text{C}$  zone and remained there for the entirety of the 5 day assay (Fig. 6). Diseased ants exhibited a change in behavior that was not observed in control cohorts. At 1600 of day 1 to the end of day 3, treated ants were not aggregated nor uniformly distributed on the gradient. On day 2, treated ants had significantly reduced numbers located in the  $<27^{\circ}\text{C}$  temperature zone compared to the control ants (Fig. 7). The largest mean separation in the percentage of exposed (47.3%a) and control ants (87.3%b) at  $<27^{\circ}\text{C}$  was at 1400 h of day 2 ( $F = 15.67$ ,  $df = 1$ ,  $P = 0.017$ ). An average of 47% and 31% of the treated ants were located at temperatures greater than  $27^{\circ}\text{C}$  on day 2 and 3, respectively. All other evaluation dates and times had an average of  $> 82\%$  of ants in the  $<27^{\circ}\text{C}$  zone for both treated and control cohorts. Average mortality of the conidia exposed replicates after 7 days was  $93\% \pm 3\%$ .

## Discussion

Evaluation of behavioral changes of *Solenopsis invicta* after exposure to *Beauveria* conidia was driven by prior laboratory and field observations of fire ant workers wandering or exhibiting erratic movements within enclosures or when climbing grass blades (Pereira and Stimac 1992, Oi and Pereira 1993, Oi et al. 1994). The description of altered behaviors associated with *S. invicta* movements and basking behavior, observed days after exposure to a pathogen, were consistent with a behavioral fever response or summit disease syndrome. Factors such as light, temperature, time and conidial dose can impact the detection of altered host behavior (de Bekker et al. 2014a). Environmental cues including both light and temperature were tested to determine if these specific cues effected individuals seeking out higher elevations during the latent infection period.

A phototaxic assay was conducted in order to evaluate if conidia exposed ants display a positive phototaxic response which could help explain movement outside of the nest and vertical movement observations. Our findings suggest that at no time point during the *Beauveria* infection process do exposed workers have a positive attraction towards light. However, the test was conducted in laboratory conditions under artificial lighting conditions. Natural sunlight, with a full light spectrum, could influence the attraction towards light; however, radiant heat from sunlight would confound potential attraction or avoidance behaviors toward lighted conditions. The lid to the arena prevented observations of wandering movements within the dark section of the arena; however, the majority of both conidia exposed and untreated ants remained within the dark region of the arena.

Increased movements of treated ants after 2 days were observed in both the mark and recapture bioassay and the heat gradient test. Observations associated with the timing of increased movement by infected workers confirm prior laboratory experiments reported by Pereira and Stimac (1992). Greatest percentages of workers observed on vertical surfaces were on day 3 and day 4. Evaluation of paint marks applied to differing body regions over time suggests elevation seeking by different infected workers and not repeated summitting events by a select few individuals.

The exact timing of dispersion and mortality can be influenced by multiple factors. Application methodology and the employment of humoral, chemical and mechanical defense tactics such as antibiotic glandular secretions, grooming, nest hygiene, avoidance, and dispersal to evade and prevent fungal infection and conidial dissemination impact the number of conidia that binds to the cuticle (Oi and Pereira 1993, Traniello et al. 2002, Konrad et al. 2012). After adherence of conidia to the cuticle, germination of *B. bassiana* conidia generally occurs between 10 h and 20 h at 20 - 25°C (Zimmermann 2007). Dependent upon the isolate, the optimum temperature for germination is 23 - 28°C with the upper range limit of 30 -38°C (Müller-Kögler 1965, Roberts and Campbell 1977).

We suggest that the lag time between exposures to conidia and wandering observed outside of a nest is predictable and not influenced by *S. invicta* social form. There was no increase in survival rates of exposed polygyne workers maintained on a heat gradient. Speed of kill and overall percent mortality was similar in all experiments. Based on increased movement in the later stages of the infection period and lack of survival in the heat gradient

choice assay, alteration of the normal host's behavior suggests a benefit to the pathogen with increasing opportunities for enhanced dissemination of infective propagules.

It can be difficult to impute the benefits of elevation seeking to either the host or the pathogen. In eusocial insects, the inclusive fitness may increase when infected individuals minimize pathogen dissemination via movement away from the colony (Evans 1989, Roy et al. 2006). However, isolating and burying infected cadavers within nest soil of *S. invicta* (Pereira and Stimac 1992, Oi and Pereira 1993), the removal of infected ants to suboptimal conditions by nestmates (Madelin 1963, Siebeneicher et al. 1992), and the antimycotic activity of venom alkaloids (Blum et al. 1958, Jouvenaz et al. 1972, Storey et al. 1991) suggests efficacious nest hygiene behaviors reduce *B. bassiana* sporulation and germination. Unless transmission is so rapid that nest cleaning rates would be ineffective, the inclusive fitness associated with the dispersal of an infected host is theorized to be unaffected (Oi and Pereira 1993). Evans and Sampson (1982) observed *Solenopsis spp.* heavily scavenging *Cephalotes* ants infected with *Cordyceps* that were securely attached to substrates; thus, summit behavior can result in greater predation or scavenging on infected hosts, thereby reducing potential pathogen dissemination rates (Roy et al. 2006).

Observations of erratic behavior in the wild are likely indicative of natural infection. Based upon the severity of exposure, the majority of directly exposed ants have increased movement between days 2 and 5 after exposure to *B. bassiana* (447). Once infected ants are found wandering outside of the mound and up vertical surfaces, mortality will often occur in less than 2 days. Applications of *B. bassiana* to colonies of *S. invicta* should be conducted in both laboratory and field settings to assess behavioral changes on a colony level and the

interactions of infected and non-infected nestmates. Based upon the time between initial exposure and behavioral changes associated with enhanced movement, weather forecast models could be used in the field to assist in the timing of application to enhance conidial dissemination. Adamo (2013) provides evidence that parasites can adaptively manipulate a host by secreting compounds that act directly on the central nervous system. *Cordyceps* spp. can alter formicids to die while biting and clinging to a vertical structure; however, carpenter ant brain manipulation by *Ophiocordyceps unilateralis sensu lato* was shown to be species-specific and seemingly associated with a close evolutionary adaptation (de Bekker et al. 2014b). We advocate that controlled studies be conducted to compare behavioral changes by *S. invicta* exposed to the highly pathogenic *B. bassiana* strains BbP89 and BbL90 that were originally isolated from an alternate host species (Chapter 1).

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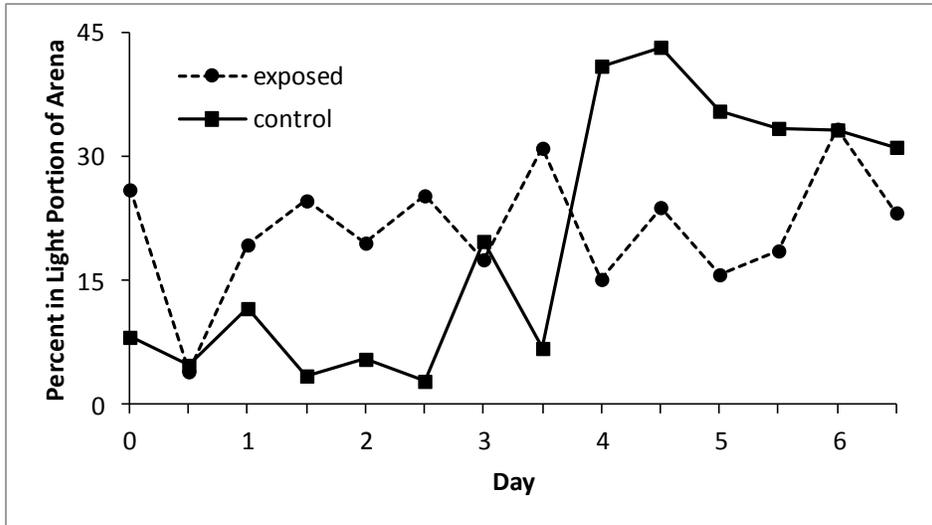
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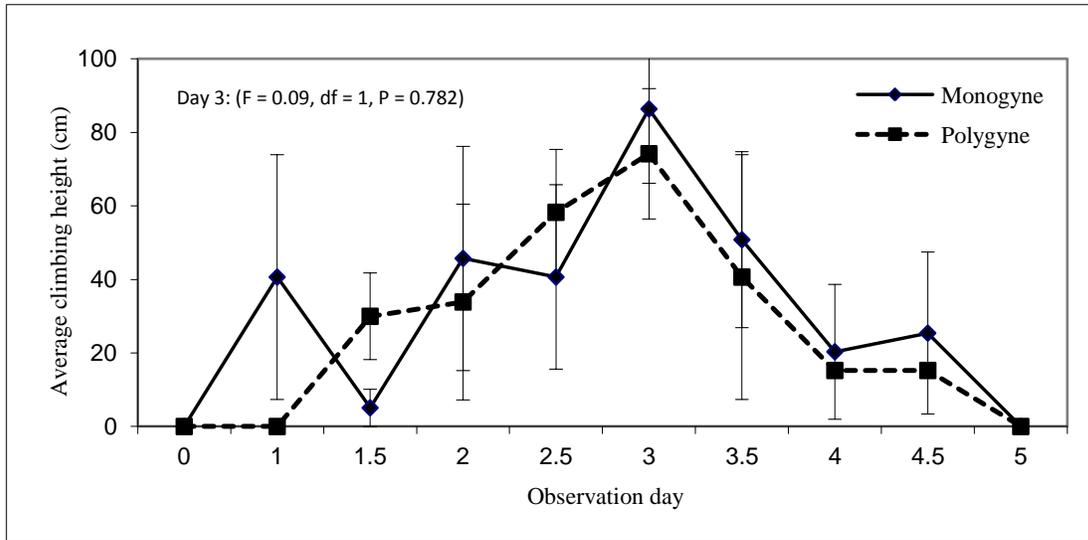


**Figure 1** Average percent of red imported fire ants ( $n = 150$ ) located in the light portion of arenas for 6 consecutive days after exposure to *Beauveria bassiana* (Bb447)

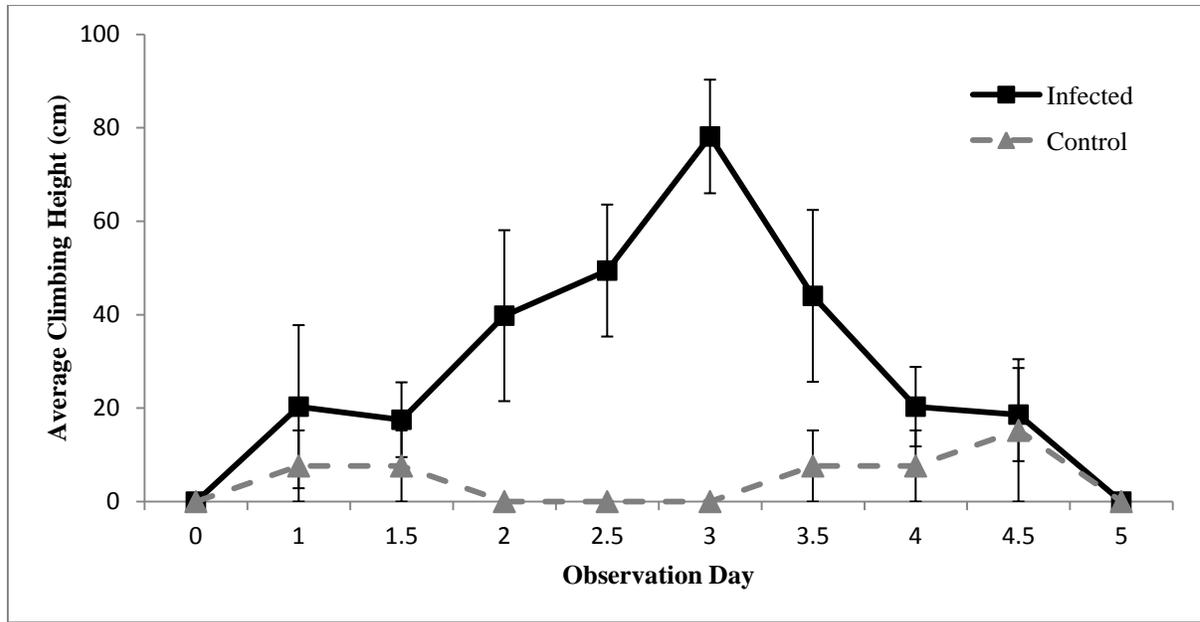
**Table 1** Mean mortality of *Solenopsis invicta* exposed to *Beauveria bassiana* (447) in phototaxis assays

Day	<u>Mean % Mortality</u>	
	Control	Exposed
0	2.7	0.0
1	3.3	0.0
2	4.0	2.7
3	4.0	29.3
4	4.0	65.3
5	4.0	82.0
6	4.0	84.7

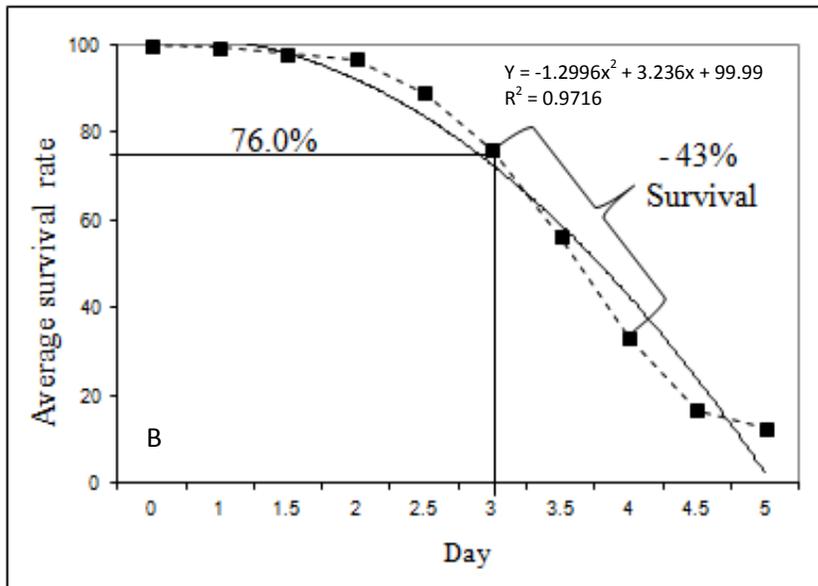
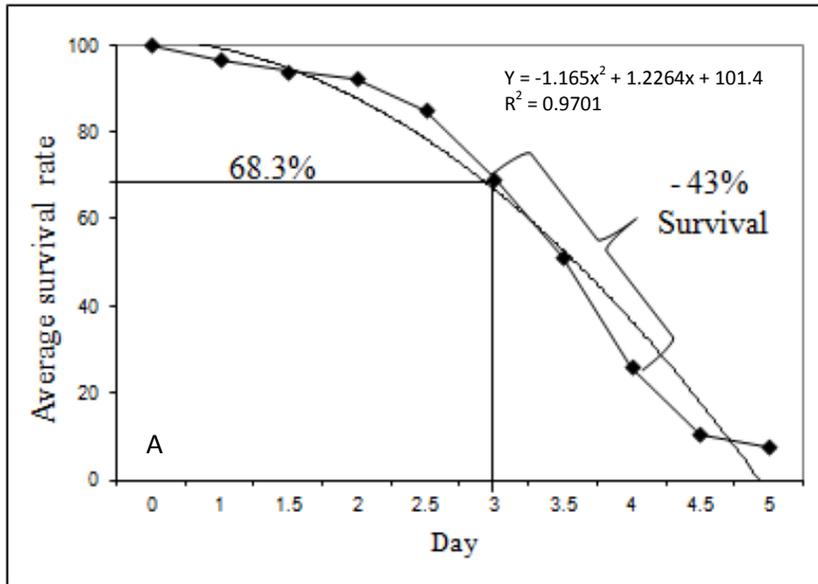
Note,  $n = 150$ ; 50 ants/treatment and the experiment was replicated three times



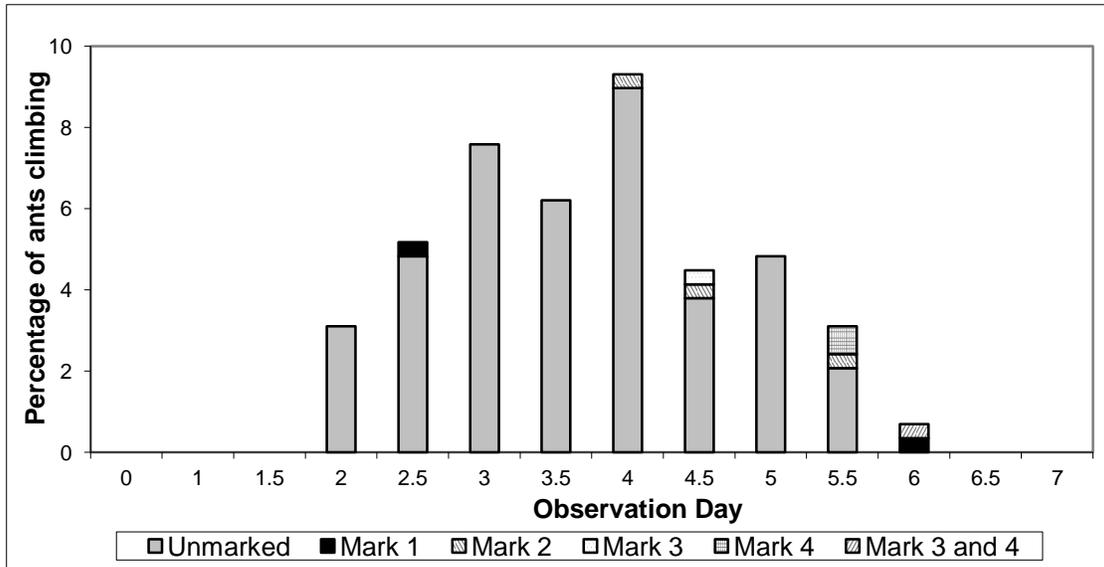
**Figure 2** Average heights of monogyne and polygyne fire ants on vertical surfaces post-exposure to *Beauveria bassiana* (Bb447)



**Figure 3** Average pooled heights of infected and control monogyne and polygyne fire ants on vertical surfaces post-exposure to *Beauveria bassiana* (Bb447)

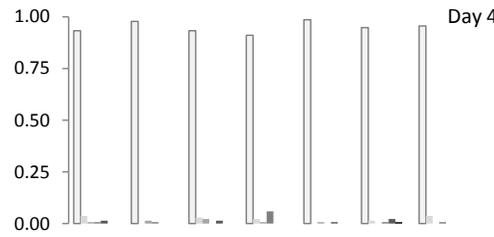
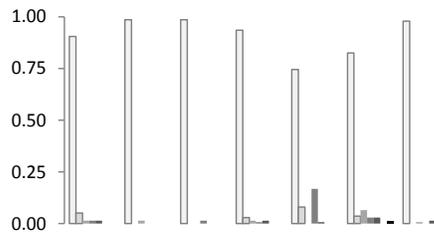
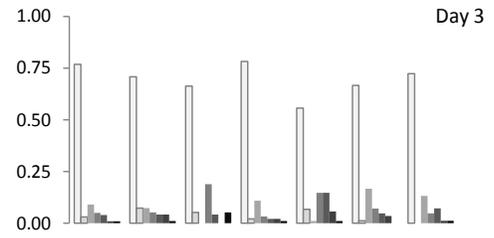
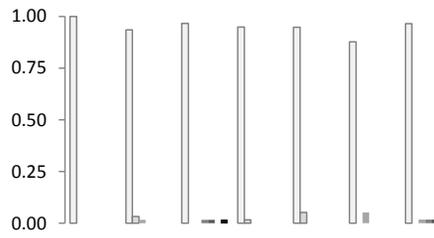
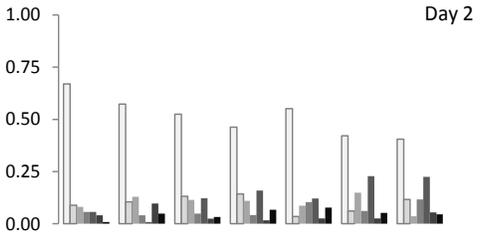
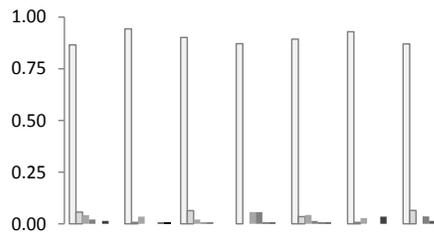
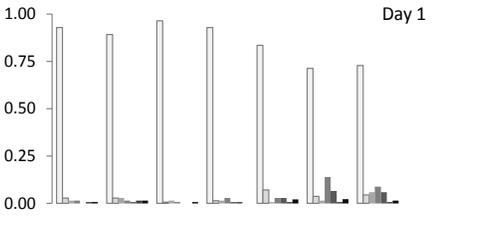
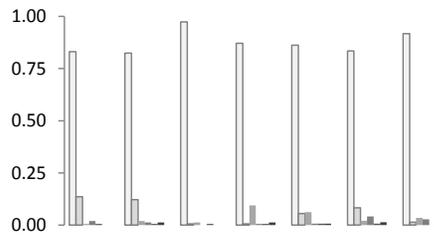
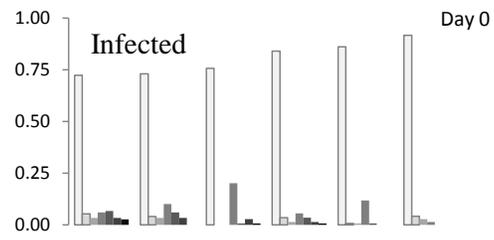
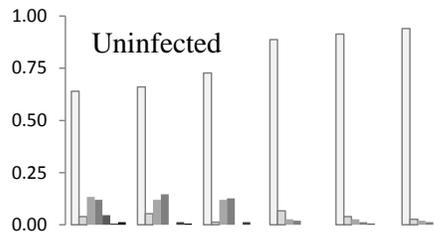


**Figure 4** Average survival rate of monogyne (A) and polygyne (B) red imported fire ants after exposure to *Beauveria bassiana* (Bb447)



**Figure 5** Average percentage of monogyne red imported fire ants exposed to *Beauveria bassiana* (Bb447) climbing vertical surfaces

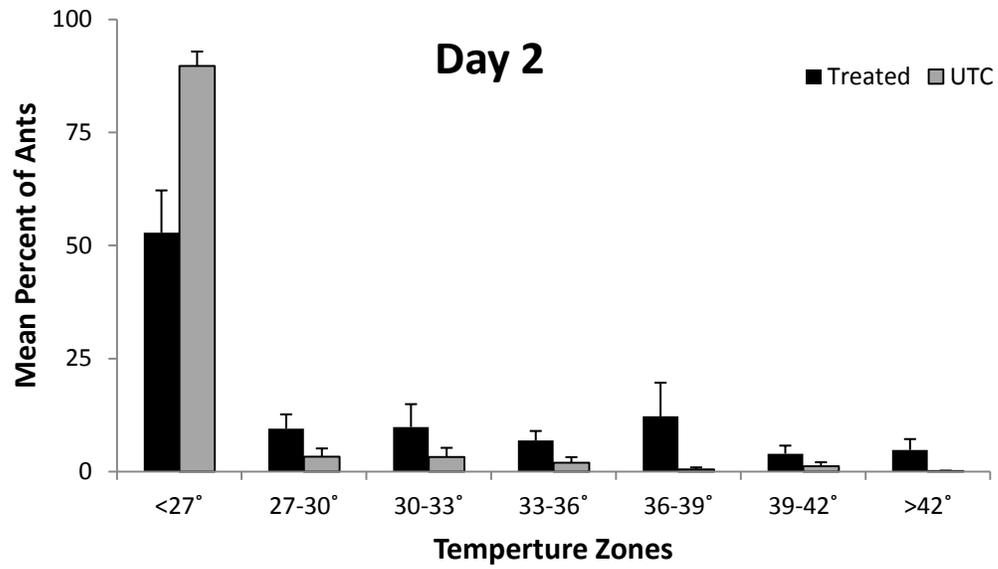
**Figure 6** Proportion of infected and uninfected red imported fire ants distributed on a heat gradient on four consecutive days after exposure to *Beauveria bassiana* (Bb447) and observed every 2 h between 0800 and 2000



8:00 10:00 12:00 14:00 16:00 18:00 20:00  
Time

8:00 10:00 12:00 14:00 16:00 18:00 20:00  
Time

□ <27°    □ 27-30°    □ 30-33°    □ 33-36°    □ 36-39°    □ 39-42°    □ >42°



**Figure 7** Pooled distributions of treated and untreated (UTC) ants on a heat gradient at two days post-exposure to *Beauveria bassiana* (Bb447)

## CHAPTER 3

Caldwell: Transfer of conidia to *Solenopsis*  
*invicta*

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**Horizontal transfer of *Beauveria bassiana* conidia and implications on survival to  
*Solenopsis invicta***

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**Abstract.** Hygienic behaviors among red imported fire ants, *Solenopsis invicta*, include the physical removal of potential pathogens, including fungi, from nest chambers. Fire ants may also clean their bodies and nestmates' bodies by self-grooming and allo-grooming using tibial combs, leg-rubbing, and licking to keep body surfaces clean. The quantity of spores found on the integument of ants exposed to *Beauveria bassiana* are reduced over time when evaluated via hemocytometer and by scanning electron microscopy. This study provides evidence of the dissemination of infective conidial propagules from exposed workers to unexposed nestmates resulting in their subsequent mortality. Individual workers were exposed to  $2 \times 10^9$  conidia / mg of dry inoculum and placed into arenas for time intervals of 5 min, 1 h, 2 h, 4 h, or 6 d; and either the treated ant remained isolated or untreated ants were introduced. Conidia were washed from the integument of treated and untreated ants at 5 min, 1 h, 2 h, and 4 h. The average number of conidia washed off of an exposed ant after 5 min was  $7.74 \pm 3.92 \times 10^5$ . After 4 h, exposed ants within multiple worker experiments had a 31.3% reduction in conidia washed from the cuticle ( $9.0 \times 10^4$ ) compared to exposed ants within the single worker experiments ( $1.31 \times 10^5$ ). All ants directly exposed to conidia prior to introduction to assay arenas were dead within 6 d. Originally unexposed ants, introduced to a conidia exposed nestmate for 4 h had mortality (4%) that was statistically equivalent to the controls. Reduced horizontal transmission from exposed to unexposed ants was not detected at other time intervals. It is hypothesized that an increased but limited duration of consistent exposure to conidia exposed nestmates enhances allo-grooming behaviors and chemical defense mechanisms which results in reduced mortality.

**Keywords.** Conidia, transfer, grooming, *Beauveria*, fire ant, *Solenopsis*

## Introduction

*Beauveria* is a genus of ascomycete fungi (Hypocreales: Clavicipitaceae) that has been demonstrated to exhibit pathogenicity in *Solenopsis invicta* (Stimac et al. 1987) with spores that disperse and make contact with the cuticle of a susceptible host via air currents, water droplets, and saprophytic growth over substrates inhabited by insects (Boucias and Pendland 1991, Holder and Keyhani 2005). Once the infective ascomycete fungal spores (conidia/ascospores) are in contact with the host, they adhere to the cuticle and germinate. During pre-germination, secretion of adhesive mucus by the swelling conidia supplements the initial hydrophobic interactions with the cuticle (Hajek and Leger 1994). The procuticle of an insect body serves as a physical barrier to fungal penetration due to the degree of thickness, tensile strength, and hardening by sclerotization (David 1967, Hassen and Charnley 1989); thus, the red imported fire ant, *Solenopsis invicta*, with heavily sclerotized body segments, are often invaded by fungal pathogens via spiracles (Charnley 1989). Once within the tracheae, thread-like hyphae called mycelia begin to grow throughout the body cavity and absorb the host's soft tissues. The host may be killed by some combination of mechanical damage or septicemia caused by the invading mycelia, nutrient exhaustion, and toxicosis (Gillespie and Claydon 1989).

Aside from normal insect immune responses (Konrad et al. 2012), ants and other eusocial insects employ chemical and mechanical defense tactics such as antibiotic glandular secretions, grooming, nest hygiene, avoidance, and dispersal to evade and prevent fungal infection and conidial dissemination (Oi and Pereira 1993, Traniello et al. 2002, Cremer et al. 2007). Fire ant venom alkaloids secreted from the poison gland will decrease conidial

germination of entomopathogenic fungi (Blum et al. 1958). *Solenopsis invicta* disperses the poison gland secretions as an aerosol via gaster flagging and it is utilized as a nest disinfectant (Obin and Vander Meer 1985). Queens from *S. invicta* colonies cover individual eggs with venom (Vander Meer 1987); workers spray developing brood and nest soil with venom which may explain the reduced efficacy of *B. bassiana* conidia when incorporated into nest soil (Obin and Vander Meer 1985, Siebeneicher et al. 1992). Furthermore, metapleural glands, which only occur in formicids (Angus et al. 1993), exhibit strong antibiotic properties and are most effective in killing fungi such as *Beauveria* and *Metarhizium* (Beattie et al. 1985). Cabrera et al. (2004) determined that the metapleural gland (MG) reservoir of *S. invicta* and *S. geminate* synthesizes carboxylic acids which are known to have antibiotic properties.

Fire ants physically remove fungal conidia from nest chambers, their bodies, and nestmates by self-grooming and allo-grooming and incorporates the use of tibial combs, the rubbing of legs, and licking to keep body surfaces clean (Wilson 1971, Holldobler and Wilson 1990). The quantity of spores found on the integument of both larval and adult *S. invicta* exposed to *B. bassiana* is reduced over time when observed by scanning electron microscopy (Oi and Pereira 1993). Another myrmicine ant, *Cephalotes atratus*, was observed to remove *Cordyceps sp.* conidia by grooming (Evans and Samson 1982). Allo-grooming has been shown to be advantageous to *S. invicta* workers and other ant species that have been exposed to entomopathogens with potential benefits to the colony via social immunity (Ugelvig and Cremer 2007, Reber et al. 2011, Qiu et al. 2014). Ingestion of inocula are often prevented because conidia are collected in the infrabuccal pocket on the

ventral surface of the buccal cavity and expelled as a pellet (Eisner and Happ 1962, Siebeneicher et al. 1992).

Dissemination of infective conidial propagules from exposed workers to unexposed nestmates during grooming events and incidental contact of *B. bassiana* 447 has not been documented in ants; however, dose transfer has been observed in termites with the entomopathogens *Metarhizium anisopliae* and *B. bassiana* (Kramm et al. 1982, Chouvenec et al. 2008, Hussain and Tian 2013). The present study was conducted in order to determine the level of *B. bassiana* conidia that are transferred from an exposed ant to unexposed ants and the resulting survival rates based upon time of exposure to the conidia exposed ant.

### **Materials and Methods**

Workers utilized in testing were taken from a single field collected and laboratory maintained *S. invicta* colony collected within one square mile of the NCSU Lake Wheeler Road Field Education Farm in Raleigh, NC (Chapter 1). The colony was confirmed via PCR to be monogyne (Valles and Porter 2003). The Bb447 strain of *B. bassiana* was originally isolated from *S. invicta* workers in Brazil and obtained from the USDA-ARS Collection of Entomopathogenic Fungus Cultures. *Beauveria bassiana* (Bb447) was selected as the test strain because it is registered for use in the United States and an estimated dose response and post-exposure behavioral modification to the host have been noted (Chapter 1, Chapter 2). Conidia were grown on rice via a modified production method and preserved at -80°C until used in the experiment (Alves and Pereira 1989, Chapter 1). The concentration of conidia per mg of dry material ( $2.9 \times 10^9$ ) was estimated visually with use of a hemocytometer.

A strain of *B. bassiana* (447) was obtained from the USDA-ARS Collection of Entomopathogenic Fungal Cultures and grown on sabouraud-maltose agar supplemented with 1% yeast extract (Anonymous 1984) and harvested utilizing the method of Watson et al. (1995). Fungi were reisolated from a second passage through ants, grown on rice, and preserved at -80°C until used in bioassays. Using a hemocytometer, a concentration of  $5 \times 10^9$  conidia/mg of dry material was determined from analyzing serial dilutions of 0.025 g of Bb447 conidia with 1mL of H<sub>2</sub>O.

Laboratory assays were initiated by placing fire ants within 9 dram vials containing autoclaved wheat flour (10 mg) and Bb447 conidia (1 mg). Capped vials were slowly agitated and remained closed for one minute during transport to the experimental arena. Arenas consisted of a 118 ml polypropylene cup with the rim coated in Fluon®. Each component of the bioassay was replicated ten times. Featherweight forceps were used to remove an individual worker ant from the treatment vial and place it within the arena; subsequently, 10 untreated workers were deposited into each arena. After a period of 5 min, 1 h, 2 h, or 4 h, the exposed ant was removed from the arena, inserted into a 1.5 ml microcentrifuge tube with distilled water and 0.05% Triton X-100 (Fisher Scientific, Fair Lawn, NJ), and centrifuged for 1 minute. Five of the 10 originally untreated ants were also removed from the experimental arena at 5 min, 1 h, 2 h, or 4 h and washed collectively. Ten replicates of a treated ant remained isolated for 5 min, 1 h, 2 h, or 4 h prior to conidia being washed from the integument were compared to treated ants within arenas that also contained untreated ants. Numbers of conidia per ml of the suspension were evaluated with a hemocytometer and microscope for both the singly exposed ant and each group of five ants.

Numbers of conidia per ant were calculated by averaging three separate hemocytometer slide counts. Scanning electron microscopy (SEM) images of a treated ant at 5 minutes post-exposure confirmed thorough conidial coverage (Fig. 1-3). The remaining 5 untreated ants were maintained within the arena until the end of the assay with mortality rates recorded daily between days 0 and 8 and bi-daily between days 8 and 20. Additionally, 10 replicates of a treated ant remained isolated or with 5 untreated nestmates for 6 d and survival assessed over the course of the experiment; these ants were not washed for conidial assessment. Positive and negative controls were included in the assay with a single treated, a single untreated (coated with flour only), or 5 untreated ants remaining isolated within an arena for 20 days. Arenas were provisioned with sugar water five hours after assay initiation.

Mortality rates and number of conidia washed from the integument were compared by analysis of variance (ANOVA) procedure ( $P = 0.05$ ) (Minitab Statistical Software v 16, Minitab Inc, PA, USA). The dissemination of conidia to untreated nestmates which resulted in mortality was analyzed using a general linear model in which the length of exposure to a treated ant was treated as the factor and the accumulated mortality was treated as the response variable. The lethal time,  $LT_{50}$ , was estimated by means of probit analysis using Minitab.

## **Results**

The average number of conidia on a treated ant at 5 minutes after inoculation, in both single worker and multiple worker experiments, did not statistically differ with  $7.74 (\pm 3.92) \times 10^5$  conidia per ant ( $F = 0.07$ ,  $df = 1$ ,  $P = 0.794$ ) (Fig. 1-3). After 1, 2, and 4 h, the number

of conidia washed off of the cuticle of exposed ants in single worker and multiple worker experiments significantly decreased (Table 1). After 4 h, exposed ants that were in the multiple worker experiments had a 31.3% reduction in conidia washed from the cuticle compared to exposed ants within the single worker experiments. Of the 10 untreated ants introduced into arenas containing a conidia-exposed ant, 5 ants were removed after 1 h, 2 h, and 4 h. Average conidial counts from washed cuticles of untreated ants did not statistically differ between sampling times ( $F = 0.85$ ,  $df = 2$ ,  $P = 0.438$ ). Ants introduced into arenas containing a treated ant and sampled at 1 h, 2 h, and 4 h after introduction had conidial counts with an average of  $4.95 \times 10^3$ ,  $6.17 \times 10^3$ , and  $6.67 \times 10^3$  conidia per ant, respectively.

All ants in all replicates directly exposed to a powder mixture of Bb447 and flour were dead within 6 days. The  $LT_{50}$  values for individually treated ants maintained within arenas with and without introduced ants were 3.46 and 3.88 days, respectively. At 6 days after exposure (DAE), significantly higher numbers of untreated ants were killed (20%) when introduced into an experimental arena with a treated ant for 1 h or continuously ( $F = 5.32$ ,  $df = 49$ ,  $P = 0.001$ ); the control replicates and ants introduced for 4 h had an average mortality rate of 4%. Mortality to ants introduced for 2 h (10%) did not significantly differ from any time variable. At 8 DAE, the rates of mortality followed a similar trend compared to 6 DAE ( $F = 4.15$ ,  $df = 49$ ,  $P = 0.006$ ). At the end of the study at 20 DAE, continued statistical differences were observed associated with average accumulated mortality between 1 h (40%a), 2 h (22%ab), 4 h (10%b), 7 d (40%a), and the negative control (8%b) groups ( $F = 7.2$ ,  $df = 4$ ,  $P = <0.001$ ) (Fig. 3).

## Discussion

No ant directly exposed to *B. bassiana* (Bb447) survived past 6 days after exposure (DAE), regardless of being isolated or paired with untreated nestmates. Reduction of conidia on the integument of the directly exposed ants was detected between 0 h and 4 h; however, the number of conidia washed from exposed ants did not statistically differ between treated workers that remained isolated or those amongst a small cohort of nestmates.

Prior to conidia adhering to the cuticle and germinating, susceptibility and transfer of viable conidia from exposed worker to an unexposed nestmate occurred. In this study, untreated ants that were introduced into experimental arenas containing a conidia covered nestmate were observed to surround the treated ant and groom the ant with their mouthparts; however, the number of conidia horizontal transmitted from exposed to unexposed ants between 1 h and 4 h did not statistically differ. Mortality was reduced and equivalent to the controls when untreated ants were within experimental arenas and in close proximity to the treated ant for 4 h (90% survival). Ants continuously exposed for 1 h or 7 days had equivalent mortality rates after 20 DAE. Workers introduced into arenas for 2 h with an exposed ant had mortality rates which did not differ from either the 1 h or 4 h groups. Direct social contact for five days with an individual previously exposed to conidiospores has led to lower susceptibility of nestmates to the same pathogen over time (Hajek and Leger 1994, Ugelvig and Cremer 2007) and lead to the upregulation of antifungal immune genes such as the antimicrobial peptides (AMP) *defensin* and *prophenoloxidase* (PPO) (Konrad et al. 2012). Social immunization has also been recently described in carpenter ants (Hamilton et al. 2011). It is hypothesized that an increased but limited duration of consistent exposure to

conidia-exposed nestmates enhances chemical defense mechanisms and allo-grooming behaviors which results in reduced mortality.

Self grooming is hypothesized to play a key role in the initial removal of conidia. A sealed mouthpart assay may provide insights on the impact of grooming with mouthparts and the implications to horizontal transmission of conidia to untreated nestmates. Increased survival has been documented within groups of conidia exposed social insects including leaf cutter ants (Hughes et al. 2002), termites (Yanagawa et al. 2010), and fire ants (Qiu et al. 2014) with direct correlations to grooming behavior and frequency (Ugelvig and Cremer 2007, Okuno et al. 2012, Qiu et al. 2014). In this study, infection and eventual mortality to the exposed worker, regardless of being isolated or with a small cohort of untreated nestmates, was likely impacted by the sheer dose of conidia; the beneficial effects of self grooming coupled with allo-grooming was prevented (Reber et al. 2011, Qiu et al. 2014).

Quantifying the number of conidia using a hemocytometer does not differentiate viable versus non-viable conidia and the utilization of chemical defense tactics to decrease conidial germination was not evaluated. Fire ant venom alkaloids secreted from the poison gland and antimicrobial compounds secreted from the metapleural gland (MG) will decrease conidial germination of entomopathogenic fungi. Although once hypothesized that MG secretions were spread passively over the cuticle, there is evidence to support that leaf cutter ants and other subfamilies selectively groom their MGs when disease agents are present. Fernandez-Marin et al. (2006) has documented the MG grooming rates significantly increase in the first hour of an *Atta* species being exposed to dry conidia and revert to baseline levels after 2-3 h of exposure. Allo-grooming could allow for more efficient spreading of

antimicrobial substances on their cuticle and ultimately reduce viable conidia and patent infection rates (Konrad et al. 2012).

Several field and laboratory studies have evaluated the efficacy of *B. bassiana* in the control of *S. invicta* with varying and inconsistent success (Siebeneicher et al. 1992, Pereira et al. 1993, Stimac et al. 1993). Different percentages of conidia treated ants within larger cohorts should be evaluated in laboratory conditions to determine if similar interactions and survival trends correlate to our observed small arena experiments or if there is an increase in colony defensive behaviors (Grace and Zoberi 1992, Chouvenc and Su 2012, Hussain and Tian 2013). Furthermore, the feasibility of utilizing the “trap and treat” method for controlling *S. invicta* with *B. bassiana* could be evaluated in the field to assess colony-level effects and the dynamics of ant-parasite interactions (Hussain and Tian 2013, Loreto et al. 2014). Future advances in biological control of *S. invicta* that uses a mound injectable formulation of entomopathogenic fungi may allow reduction in *S. invicta* populations without the necessity to use synthetic chemistries for individual mound treatments; however, consideration of an innovative formulation that disrupts grooming and allo-grooming events without negatively impacting horizontal transmission of conidia to untreated nestmates should be considered.

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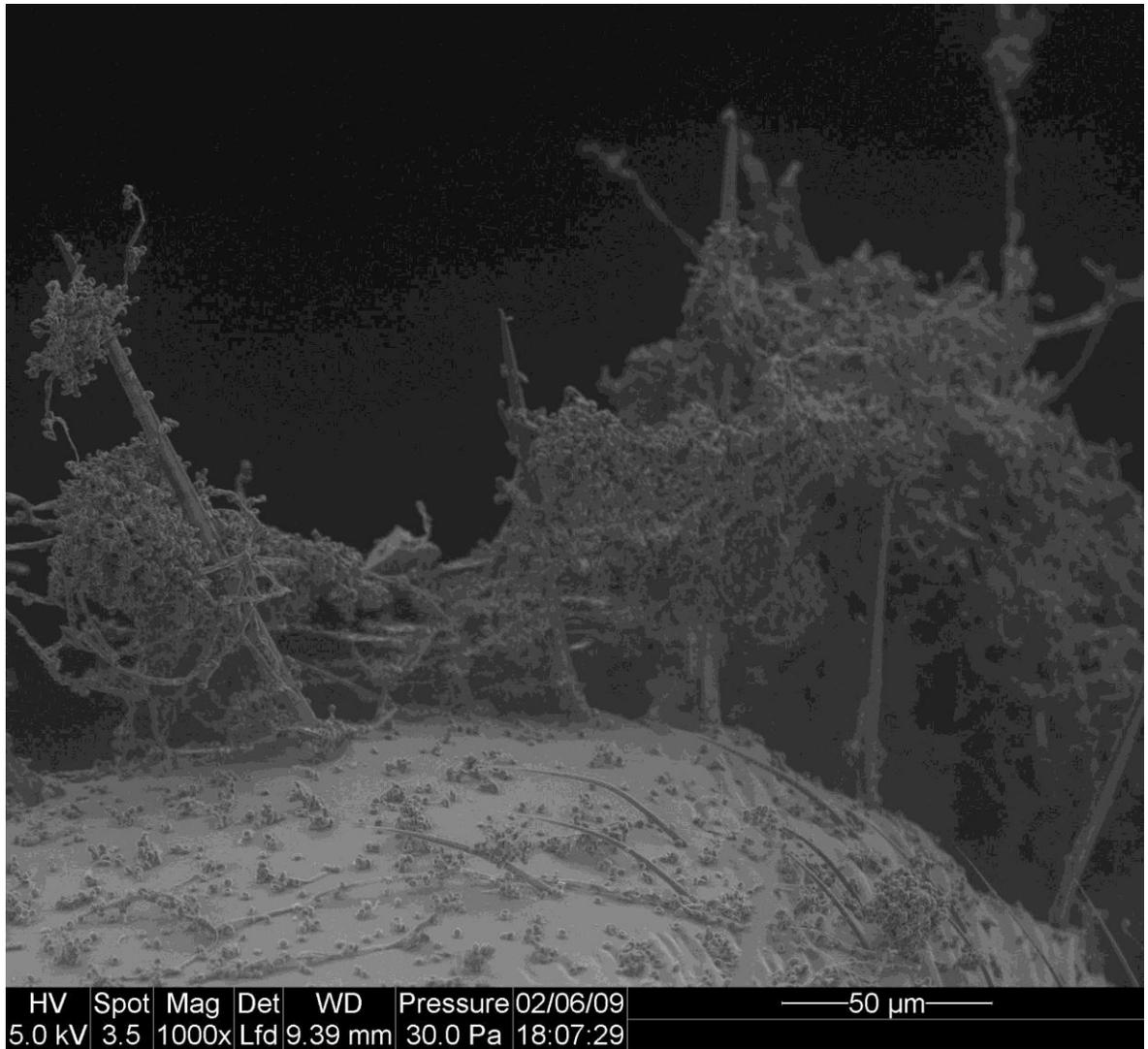
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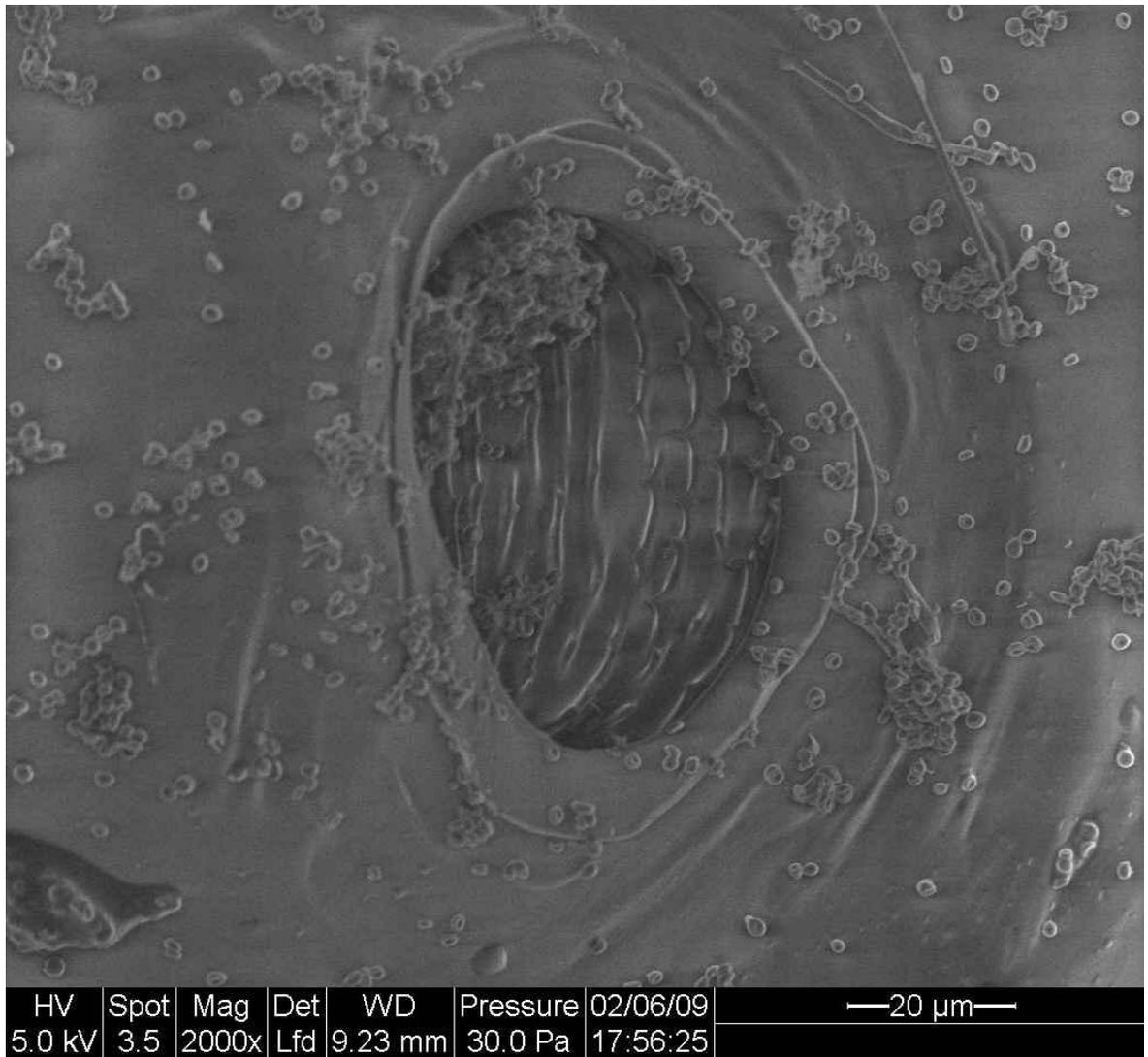
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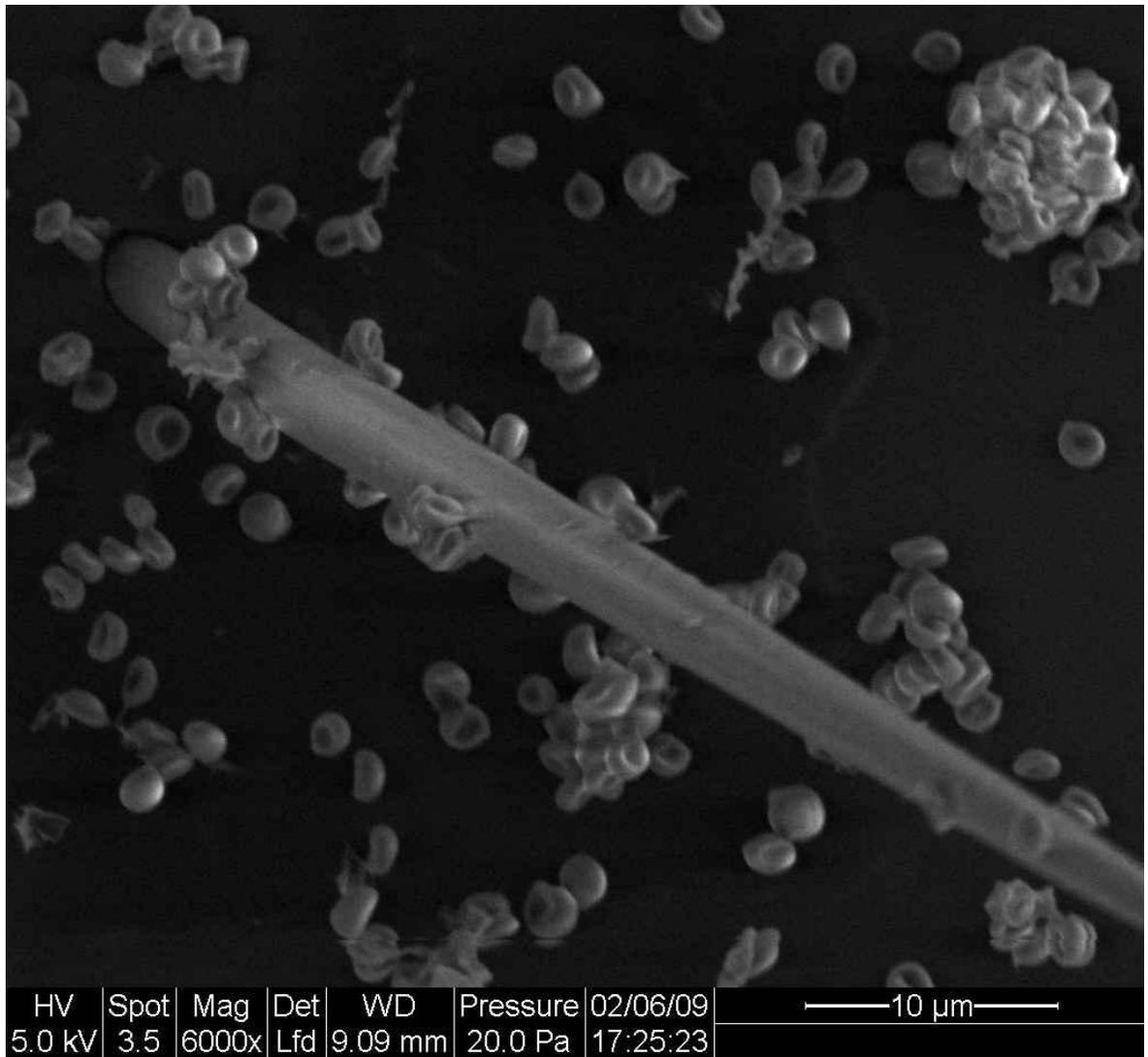
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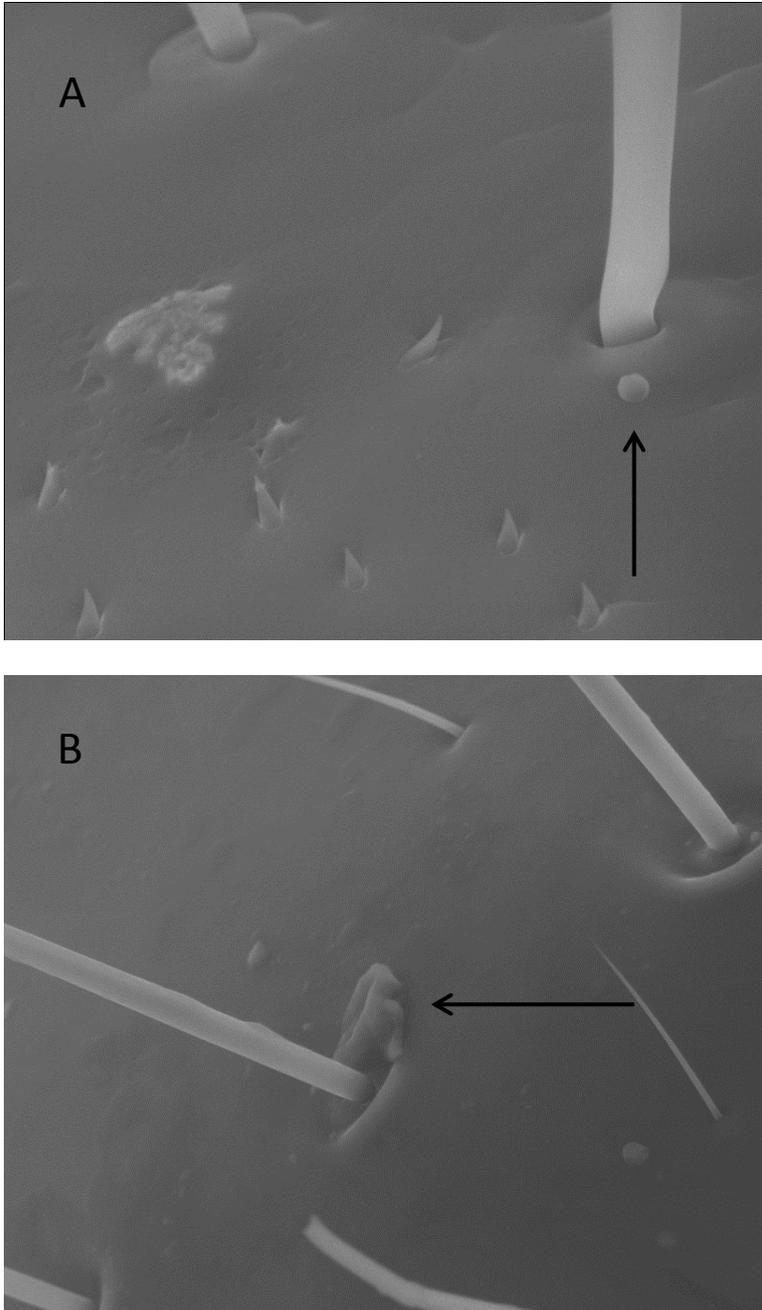
**Figure 1** SEM of conidia on the integument of the mesonotum of a *S. invicta* worker exposed to *Beauveria bassiana* (Bb447) conidia for 5 minutes



**Figure 2** SEM of conidia on the integument of the thoracic spiracle of a *S. invicta* worker exposed to *Beauveria bassiana* (Bb447) conidia for 5 minutes



**Figure 3** SEM of conidia on the integument of an abdominal seta of a *S. invicta* worker exposed to *Beauveria bassiana* (Bb447) conidia for 5 minutes

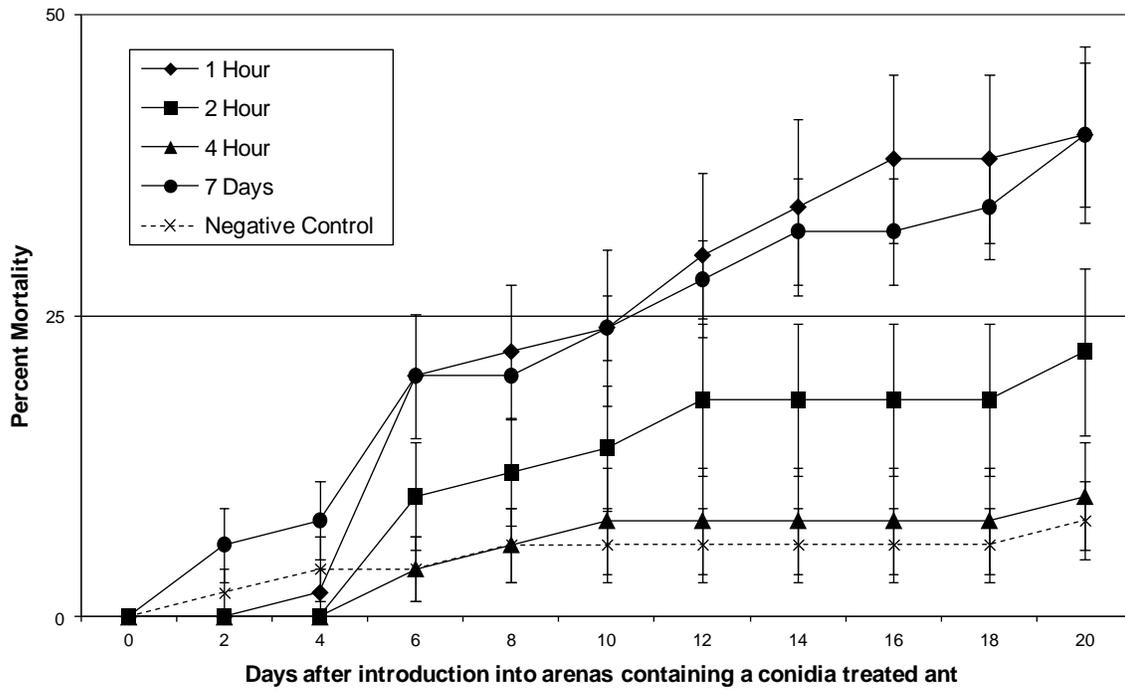


**Figure 4** SEM of a *Beauveria bassiana* (Bb447) conidium directly adjacent to an abdominal seta at 30 minutes (A) and 72 h (B) after exposure to a *S. invicta* worker

**Table 1** Average number of *Beauveria bassiana* (Bb447) conidia washed from a single exposed ant after allo-grooming or self-grooming for 5 min to 4 h after exposure

Grooming type	Time after exposure	Average number of conidia
Allo-groomed	5 minutes	$7.00 \times 10^5$ a
Self-groomed	5 minutes	$6.73 \times 10^5$ a
Allo-groomed	1 hour	$3.00 \times 10^5$ b
Self-groomed	1 hour	$1.88 \times 10^5$ bc
Allo-groomed	2 hour	$2.46 \times 10^5$ bc
Self-groomed	2 hour	$1.47 \times 10^5$ bc
Allo-groomed	4 hour	$9.00 \times 10^4$ c
Self-groomed	4 hour	$1.31 \times 10^5$ bc

Note: Means associated with column are not significantly different; Tukey; 95% CI.



**Figure 5** Average cumulative percent mortality ( $\pm$ SE) of red imported fire ant cohorts after introduced into an experimental arena containing a single ant exposed to a powder formulation of *Beauveria bassiana* (Bb447) for varying lengths of time