HOST/PARASITE INTERACTIONS BETWEEN

Solenopsis invicta (HYMENOPTERA: FORMICIDAE) and

Thelohania solenopsae (MICROSPORIDA: THELOHANIIDAE)

A Thesis

by

MICHAEL WALKER HALE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Entomology

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Approved by:

Chair of Committee,	S. Bradleigh Vinson
Committee Members,	Julio S. Bernal
	Markus J. Peterson
Head of Department,	Kevin Heinz

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ABSTRACT

Host/Parasite Interactions Between *Solenopis invicta*

(Hymentoptera: Formicidae) and *Thelohania solenopsae*

(Microsporida: Thelohaniidae). (May 2006)

Michael Walker Hale, B.S.; B.S., Texas A&M University

Chair of Advisory Committee: Dr. S.B. Vinson

Thelohania solenopsae Knell, Allen and Hazard is a microsporidian pathogen that infects the red imported fire ant *Solenopsis invicta* Buren. This five part study examined the effects that *T. solenopsae* has on constructs of colony fitness in field mounds and adoption rates, how *T. solenopsae* spores are affected by different stable temperatures. This study also examined the effects on *T. solenopsae* spores due to centrifugation out of the host cell, pH of the solution the spores are kept, and food classes that could be ingested by *S. invicta*. For the first study, a total of 29 colonies were collected, 16 infected, and 13 uninfected. The study concluded significantly lower brood production in uninfected field colonies when compared to infected field colonies. Additionally, the total number of queens per colony is significantly greater in *T. solenopsae* infected mounds with 35.4±31.9 queens for infected mounds and 15.5±11.2 queens for uninfected mounds, demonstrating a possible host response to parasite infection. In the study examining stable temperature effects on *T. solenopsae* spore growth and spore type, a stable temperature of 31˚C induced greater production of binucleate free spores in worker *S. invicta*. There was an overall decrease of *T. solenopsae* octospores at 16˚C, 26˚C and

31˚C. The studies concerning extraction of *T. solenopsae* spores, and inclusion in food infusions demonstrated viable spore recovery after centrifugation, verified by using Calcofluor M2R and Sytox Green dual staining. This study demonstrated significant decreases in spore viability over a 10 week period. The study concerning pH demonstrated significant effects of differing pH parameters with the best viable spore recovery at pH 4 and no spore recovery at pH 1 and pH 2. The least viable spore recovery occurred at pH 6 and pH 12 after 24 hours of emersion verified by using Calcofluor M2R and Sytox Green dual staining.

DEDICATION

I dedicate this to my parents who supported any endeavor that I had, no matter how unusual it may have been. Thank you for giving me the greatest gift of all, life, and the second greatest gift, curiosity. I also dedicate this to my friends, or as I refer to them my second family. You fulfill my need to be part of a society, much like the ants I study.

ACKNOWLEDGEMENTS

There are so many people I would like to thank for their assistance in completion of my thesis. First, thank you to my advisor, Dr. S. Bradleigh Vinson, for all the insights of a seasoned scientist, and for giving me the space and patience needed to actualize this work. Thank you to Dr. Bernal and Dr. Peterson for the work you both have done in corrections and making all the deadlines. Thank you to the Texas Fire Ant Research and Management Initiative for funding, which means directly thank you to the people of Texas, your money is being put to good use.

An extra special thanks to Claire McKenna, for being both technical and emotional support. You made everything run much smoother.

TABLE OF CONTENTS

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae), an exotic species, has become a serious medical and agricultural pest in the southeastern United States (Lofgren, 1986). This pest poses direct and indirect urban, agricultural, wildlife, industrial, legal, and political problems (Vinson, 1997). By 1995, the red imported fire ant, (RIFA), expanded its range to 114 million ha in all or part of 670 counties/parishes in 11 states and Puerto Rico (Callcott and Collins, 1996), and recently invaded California (Jetter et al., 2002). RIFA has become a dominant arthropod species and has reduced biological diversity in the areas it infests due to its high reproductive capacity, aggressive foraging behavior, and lack of effective natural enemies in the United States (Porter and Savignano, 1990).

Chemical control has been the main management approach toward RIFA, but this method is not economically feasible in most agricultural and wildlife regions where RIFA has significant impacts (Drees and Vinson, 1993). Also, chemical control affects non-target, native ants (Summerlin et al., 1977). Biological control is a non-chemical management alternative for RIFA control using natural enemies, including pathogens, to keep populations at an acceptable or non-damaging level. Surveys have been undertaken in the last few decades to identify RIFA biological control agents in the southern United States and South America for use in biological control of RIFA (Jouvenaz et al., 1977, 1981). Porter et. al (1992) in a census of mound densities found that RIFA were much more abundant in the United States than in South America.

This thesis follows the format of the Journal of Invertebrate Pathology.

This finding supported the hypothesis that RIFA was introduced without any of its important natural enemies (Jouvenaz, 1983, Porter et al., 1997).

Among the biological control agents discovered thus far, microsporidia have shown some promise as a bio-pesticide (see Brooks 1988, for review). Microsporidia are unicellular, obligate intracellular parasites of eukaryotes. They are unusual, in that they lack mitochondria, peroxisomes, and other typical eukaryotic structures (Keeling et al., 1998).

The microsporidium *Thelohania solenopsae* Knell, Allen and Hazard, (Microsporida: Thelohaniidae) was first discovered in RIFA by Allen and Buren (1974) in alcohol-preserved specimens collected from Mato Grosso, Brazil, and first described by Knell et al. (1977). In the United States, Williams et al. (1998), first found *T. solenopsae* in RIFA workers along a roadside (US 441) by Payne's Prairie 8 km south of Gainesville, Florida. Its discovery in Florida facilitates research on *T. solenopsae* because it precludes the need for quarantine handling, which is required of exotic species considered for use in biological control. Moreover, RIFA individuals infected with *T. solenopsae* have been recovered from south central Texas field sites (Chen et al., 2004).

The research objectives for this study were designed to examine several aspects of the host parasite relationship between RIFA and the microsporidium *T. solenopsae*. Since *T. solenopsae* is a potential biological control agent of RIFA, an understanding is needed of the interactions between the two species, as well as methods for its culture in the laboratory.

The first objective was to examine the effect *T. solenopsae* may have on field collected populations of polygyne RIFA in reference to brood mass, numbers of dealated

reproductives, and the insemination status of the dealated reproductives. In RIFA, only the dealated reproductive can produce eggs (Fletcher and Blum, 1981) and thus contribute to the genesis of the colony. Furthermore, only inseminated reproductives can contribute to the worker caste. Comparisons of contributing reproductives and brood production quantified fitness effects on RIFA due to *T. solenopsae* infection. A follow up study compared adoption rates of introduced RIFA queens between *T. solenopsae* infected and uninfected colonies to further discern potential host response to infection by this pathogen.

The second objective was to examine the effects of temperature on *T. solenopsae* production within its host, and whether spore proliferation, or spore type could be affected by different constant temperatures. Currently, there is no culturing technique for growing spores other than maintaining colonies of the *T. solenopsae*-infected RIFA. If specific temperature regimes differentially elicit production of particular spore types, this knowledge can be used to save time and space needed for culturing *T. solenopsae*.

The third objective of this study was to utilize modified centrifugation techniques and assess spore viability after centrifugation. After centrifugation, spore viability was monitored over time when infused into different classes of food ingested by RIFA. Furthermore, pH gradients were used to determine if there are optimal pH parameters for spore activation or spore storage.

CHAPTER II

REVIEW OF LITERATURE

Microsporidia have played an important role in the history of microbiology. The microsporidium *Nosema bombycis* Naegli, first described by Louis Pasteur in 1870, proved to be the causal agent of *pebrine* disease in the silkworm *Bombyx mori* L. (Boucias and Pendland, 1998). Pebrine disease was among the first insect diseases to be documented and studied by early microbiologists. Study of microsporidia historically focused on the prevention of microsporidiosis in cultured stocks of insects and fish, though now microsporidia are now considered emerging pathogens because of new species identified as causes of human diseases during the last 20 years (Boucias and Pendland, 1998). Currently, a product is commercially available that uses *Nosema locustae* Canning, as a microsporidiosis causing agent to combat grasshopper populations (Lomer and Langewald, 2001).

Though all microsporidia are parasitic, their biology and structure are extremely varied due to their relationships with the host organism, or organisms. After coming in contact with the appropriate host cell, usually midgut epithelium and fat bodies, microsporidia enter by extruding a coiled polar filament that penetrates the host cell, and microsporidial sporoplasm is passed into the cell, initiating a new infection (Müller, 1997). Infections in insects have mostly chronic or sub-lethal effects, and generally there are no outward signs of parasitization or infection (Becnel and Andreadis 1999). Studies on *Solenopsis* microsporidia are in preliminary stages. Field studies on populations of *Solenopsis richteri* Forel in Argentina indicated that low densities of this ant species were associated with the presence of microsporidia (Briano et al., 1995).

Briano and Williams (1997) showed that mortality rates of *S. richteri* workers from infected colonies between 8.8 and 29.2% greater than those of healthy workers at 27ºC. Williams et al. (1999) reported that brood production was significantly reduced and queens died prematurely in laboratory infected monogyne colonies.

T. solenopsae has been primarily found infecting the polygyne form of RIFA in the field, although monogyne colonies have been infected in laboratory experiments (Williams et al., 1999). The reasons for prevalence of infections in polygyne versus monogyne colonies are unknown, but may include premature death of monogyne queens, and therefore a lower frequency in field samples, or specific innate differences in social interactions within and between polygyne and monogyne colonies. One genetic mutation at the Pgm-9 locus creates the polygynous social form known to have multiple mated queens contribute to reproduction, and lack of aggression in the worker forms regardless of colonial origin (Keller and Ross, 1999). Other specific difference between the monogyne and polygyne forms are the ability of polygynous colonies to produce new colonies by colony fission (Tschinkel, 1998), and that their collective populations, may be 2-3 times greater than those in monogyne infested areas (Vinson 1993, Macom and Porter, 1996).

Oi and Williams (2002) reported that brood levels in *T. solenopsae* infected polygyne colonies declined to nil after 26-52 weeks, even in infected colonies with fertilized, uninfected queens. Vegetative stages of *T. solenopsae* have been found in eggs, and thus shown to be transovarially transmitted (Briano et al., 1996). Available data suggests that the life cycle of a microsporidium is correlated to the life cycle of the host ant, with vegetative stages found in ants from the egg up to pupal stage, and spores in

pupal and adult stages (Briano et al., 1996).

Many of the characteristics of *T. solenopsae* make it a good potential candidate as a biological control agent. Anderson (1982) outlined optimal characteristics of pathogens for their use as biological control agents:

1) An intermediate pathogenicity combined with the ability to reduce host reproduction. *T. solenopsae* produces an infection that causes an overall weakening of the colony, increased mortality and reduced fecundity (Williams et al., 1999);
2) High transmission efficiency. *T. solenopsae* has both vertical and horizontal transmission routes. Infected queens lay infected eggs. The mechanism of horizontal transmission is not completely understood, but a healthy colony can be infected via infected brood from an infected colony (Williams et al., 1999, Oi et al., 2001);
3) The ability to produce large numbers of long-lived infective transmission stages. In microsporidia, spores are the stable transmission stage. Spores are able to resist loss of host contact, and desiccation (Maddox, 1973).

These factors make *T. solenopsae* a good candidate for further investigations concerning its use as a biological control agent for RIFA.

CHAPTER III

Thelohania solenopsae EFFECTS ON *Solenopsis invicta* FIELD COLONIES

Introduction

Social life does not exclusively provide benefits, but also entails costs such as increased vulnerability to pathogens and parasites (Keller, 1995). Physical proximity and contact rates increase in groups, thereby increasing the likelihood that individuals will encounter parasites or pathogens introduced by other group members. Moreover, pathogens may be one of the factors accounting for the association between queen number per colony and the presence/absence of polymorphic workers in ants (Frumhoff and Ward, 1992, Keller, 1995). Through analysis of field samples, this study tested the hypotheses that *Thelohania solenopsae* infection had no effect on RIFA brood mass, dealated female reproductive numbers, their insemination status, and worker size composition, in field collected RIFA colonies. Additionally, this study also addressed whether potential queens differ between T. solenopsae infected and healthy colonies.

Materials and Methods

RIFA colonies were collected from the vicinity of the Animal Science Center, TAMU, during the spring of 2002 and of 2003. This area was known to harbor RIFA colonies infected by *T. solenopsae*, (Chen et al., 2004). Colonies were dug out from their ground locations and placed into 19 L buckets. Buckets were dusted with talcum powder around the inner circumference to prevent escape of ants. The RIFA were then dripped out using the method of Banks et al. (1981), and placed in 16”×10¾”×6½” (40.64cm× 27.31cm× 16.51cm) plastic boxes (Stackable Storage Box First Phillips Marketing, Leominster, Mass). The boxes were coated with Fluon® (AGC Chemicals Americas

Inc., Bayonne NJ) to prevent escape of ants. The colony nest consisted of a Falcon® Petri dish (150mm×15mm) half filled with Castone® (Dentsply, Ft. Worth, TX) plaster. Colonies were then typed as positive or negative for *T. solenopsae* infection by macerating 25 worker ants in 200µl of water using a Mini Bead Beater® and 0.5mm beads in (Cole-Parmer Instrument Co., Vernon Hills IL) 2.0 ml vials for Mini Bead Beater®. A 20µl sample of the supernatant was examined under 400× magnification with a Zeiss® phase-contrast light microscope to screen for meiospores, single nucleate free spores derived from meiospores, or binucleate free spores. Modified trichrome staining (Weber et al., 1992) was used to cross check infection status.

All dealates (n=263) from 13 colonies collected in 2002 were individually placed in 2.0 ml Mini Bead Beater® vials labeled, and frozen at -20°C. Dealates were then individually examined under a dissecting scope to ascertain insemination status by the presence or absence of a pearl white spermatheca. Then, each dealate was ground using a Mini Bead Beater and 0.5mm beads in 200µl of water and screened under 400× magnification for the presence of binucleate free spores. Brood consisting of eggs, 1^{st}-4^{th} instar larvae, and pupae were separated from the colony by CO_2 narcosis of the workers, and weighed using a Mettler® AE240 balance. Samples of workers were randomly gathered from each of the colonies in 2002, and head widths of the first 30 were measured under a dissecting scope for subsequent analysis. Head widths of RIFA have been used to calculate overall ant size because of the close correlation of head capsule width and total body mass in RIFA (Tschinkel et al., 2003).

The number of total queens per brood was scored per colony independent of infection status. Independent samples *t*-tests were performed to determine if there were

statistical differences in brood mass, and queen number between *T. solenopsae* infected and uninfected colonies. All data were analyzed using SPSS version 12.0 (SPSS, Inc., Chicago IL, 2001). An independent samples *t*-test analysis for worker head widths was performed to determine if there were any significant difference in *T. solenopsae* infected or uninfected colonies. A χ^2 Goodness of Fit test was used to determine if the frequency of infection differed between inseminated and uninseminated dealates within *T. solenopsae* infected colonies.

A follow up study was conducted with 25 colonies collected in 2003 to compare adoption rates of introduced intercolonial queens between infected and uninfected colonies. Sub-colonies were created using five queens, two grams of brood, and two grams of workers per colony. All sub-colonies were contained in an individual shoe box 12 ½" × 6 ¾" × 3 ¾" (31.75cm×17.15cm× 9.53cm) (First Phillips Marketing Stackable Storage Box, Leominster, Mass) in nests consisting of 100 ×15mm Falcon® Petri dish half filled with Castone® (Dentsply Ft. Worth, TX) plaster to retain moisture. Ten sub-colonies positive for *T. solenopsae* and 10 sub-colonies negative for *T. solenopsae,* were allowed to establish for two weeks. After two weeks, there was a one-time introduction of five queens into each sub-colony. These queens were derived from established colonies whose workers screened negative for *T. solenopsae,* and were visually pre-screened to avoid introduction of damaged queens, such as queens with broken antennae, or abdominal wounds. Moreover, introduced queens were established queens from extant polygyne colonies because Vander Meer and Porter (2001) demonstrated that newly dealated flight queens were killed at a rate of 100% in established polygyne mounds. Also, newly emerged dealates occur infrequently within a collecting season. Queen

mortality per colony was recorded at 72h post introduction, and compared between *T. solenopsae* positive and negative colonies using a *t*-test.

Results

The total brood mass was about one third greater in healthy colonies compared to infected (t=-2.206, df=27, P=0.033) (Fig 1). In contrast, the number of queens per colony was significantly greater in *T. solenopsae* infected versus healthy mounds (t=2.345, df=19.349, P=0.03) (Fig. 2). In addition, queen reproductive output was greater in healthy colonies (1.2gm/queen ±1.0) compared to *T. solenopsae* infected colonies (0.5 gm/queen ±0.3gm) (t=-2.419, df=13.728, P=0.03).

The mean head capsule width of ants from uninfected colonies was (0.623mm ± 0.120 mm) and did not significantly differ from that of ants from infected colonies (0.609mm ± 0.124mm) (t=-0.820,df=212, P=0.413).

The rate of *T. solenopsae* infection of individual dealates was significantly higher in inseminated versus uninseminated dealated females (χ^2=6.39, df=1, P<0.025) (Fig.3).

In the follow up study, a significantly smaller number of queens survived after 72 h post introduction into healthy colonies (t=2.563, df=17.42, P=0.02) (Fig. 4).

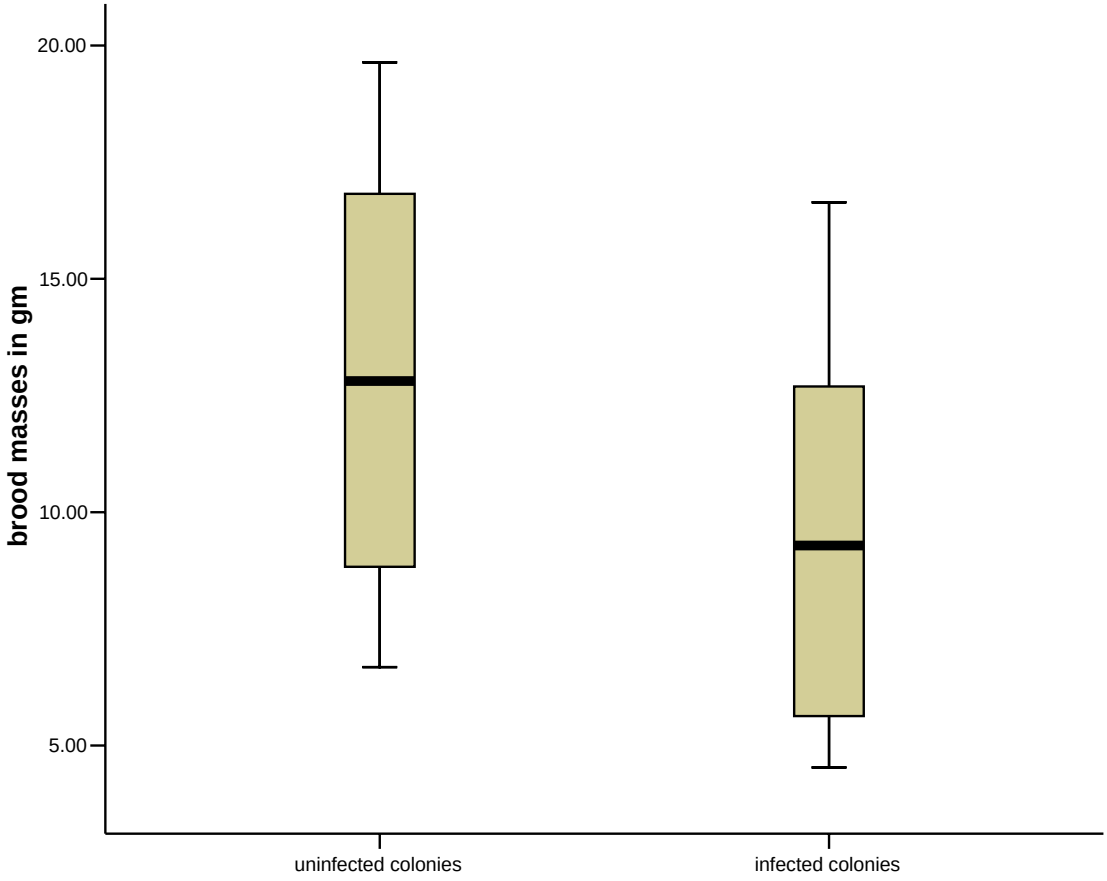

Fig. 1 Comparison of brood masses between *T. solenopsae* infected and uninfected colonies. Whiskers represent lower and upper quartile. Box edges are second and third quartile. Black lines represent the medians. P= 0.033

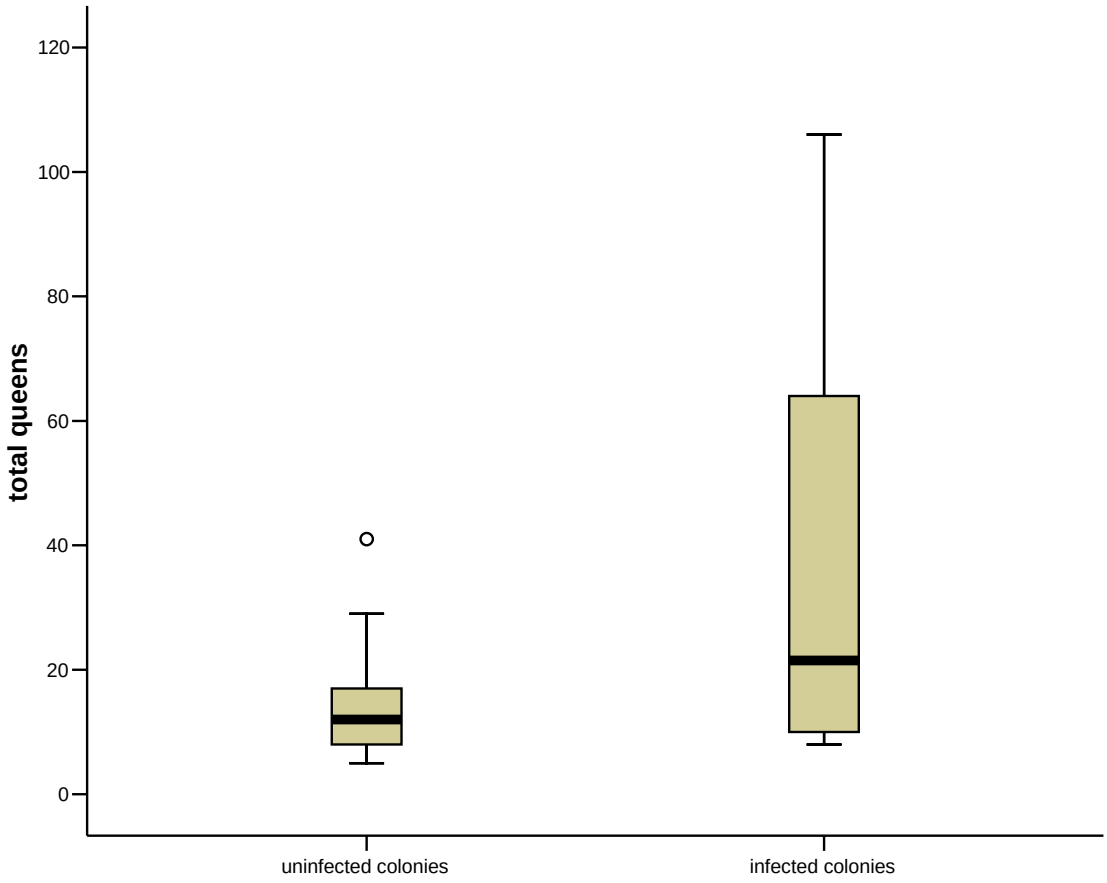

Fig. 2 Comparison of total queens found in *T. solenopsae* infected and uninfected colonies. Whiskers represent first and fourth quartiles. Boxes denote second and third quartiles. Black lines represent the medians. Circles represent mild outliers in data. P= 0.03

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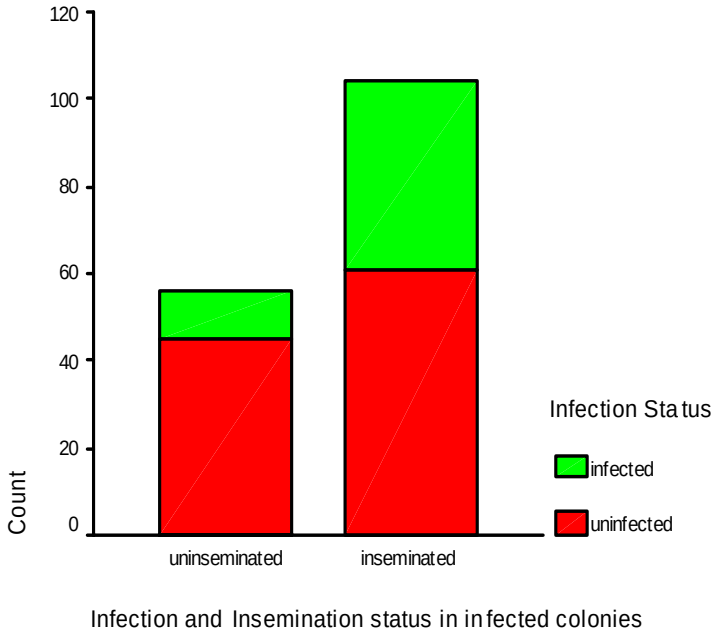

Fig. 3 Infection rates of dealate inseminated and uninseminated reproductives from *T. solenopsae* infected colonies collected in 2002. $P<0.025$

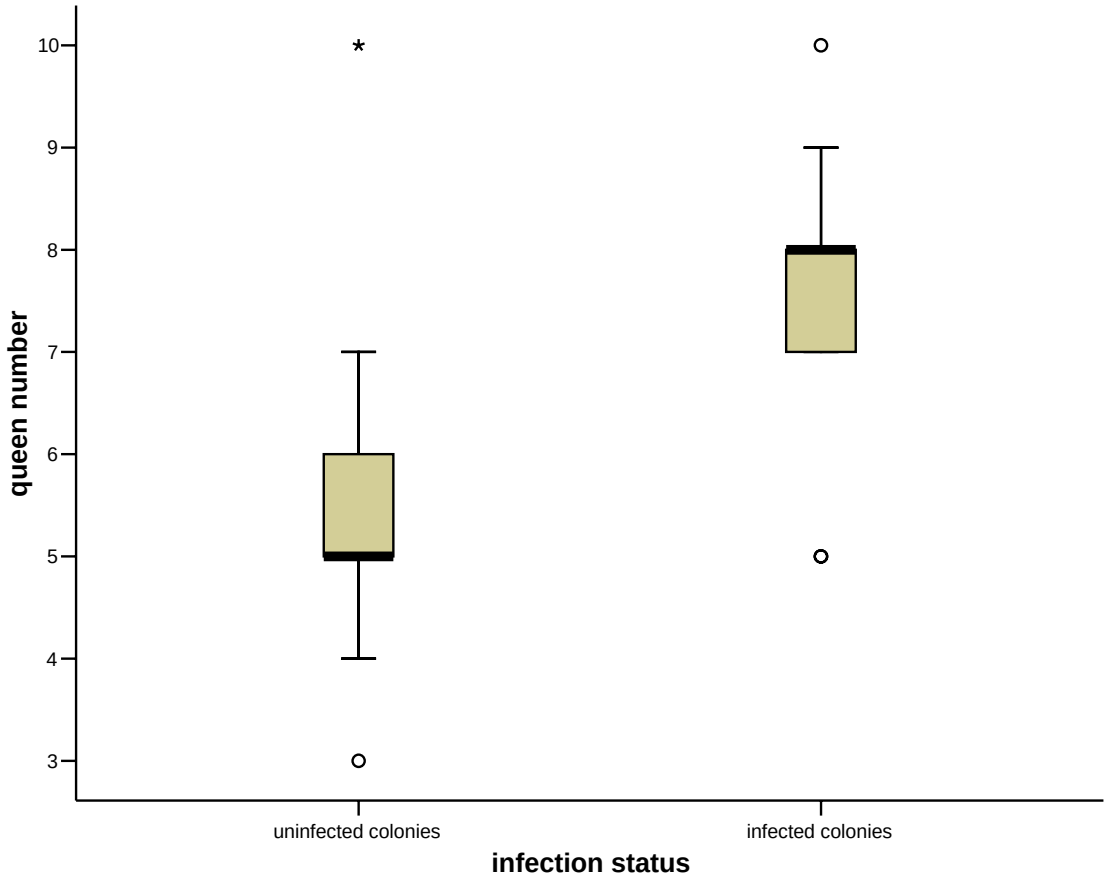

Fig. 4 Queens alive 72 hours post introduction of established queens into *T. solenopsae* infected and uninfected colonies. Whiskers represent first and fourth quartiles. Box represents first and third quartiles. Black lines represent the medians. Circles are mild outlying data. Star represents extreme outlying datum. P= 0.02

Discussion

Brood mass, insemination status, queen number per colony, and worker head widths were used as indices to determine if *T. solenopsae* infection had an effect on colony fitness. Specifically, these indices were used to assess if *T. solenopsae* could affect queen numbers in the polygyne colony, brood masses in field mounds, insemination rates of dealated females, and worker polymorphism. The results of this study showed that field collected, *T. solenopsae* infected colonies had a greater mean number of queens per colony, had lower brood mass, and lower queen to brood ratio compared to uninfected colonies.

The difference in queen number could be explained by several possible factors. In one scenario, dealated reproductives could be over-represented in infected mounds due to the inability of a *T. solenopsae* infected alates to successfully initiate or complete a mating flight (Overton et al., in press). However, the results of this study showed that infection rates were higher among inseminated versus uninseminated dealated reproductives, and does not support a hypothesis in which *T. solenopsae* infected queens resulting from dealated reproductives remain in the mound. Differential executions of dealates within polygynous colonies, where cooperation and lack of aggression are the norm (Vinson, 1997), have been explained as due to genetic differences (Keller and Ross, 1999, Goodisman et al., 2000) and relative age of the dealate (Vander Meer and Porter, 2001). Chen and Vinson (2000) demonstrated feeding and movement preferences of the largest, most reproductive queens, and Overton et al. (in press) demonstrated significantly lower lipid stores in *T. solenopsae* positive alates. Under the adverse condition of increased temperature, queen preference on the basis of size has also been

demonstrated (Kuriachan and Vinson, 2000). It is possible that infected, non-reproductive females are passively or actively executed as suggested by the lack of dealated infected, uninseminated RIFA. As colonies need to maintain brood for processing proteins or solid foods into a liquid form that worker ants can ingest (Sorensen et al., 1983), dwindling brood numbers lead to a diminished ability to utilize food, and colony fitness decreases. This necessary bio-feedback loop, through chemical cues or hunger, may elicit a response to add more reproductives to a mound that is losing brood due to the effects of *T. solenopsae* infection. Second, infected workers or the extant queens may be less aggressive towards new reproductives entering the nest, leading to fewer executions, and accumulation of reproductives in the mound. Although the above scenarios are not mutually exclusive, the second is supported both by the significantly lower adoption or retention rate success in healthy colonies, and evidence that *T. solenopsae* infected colonies are less aggressive towards invading native ants (Molly Keck, personal communication). But it is not known if the effect of accumulating queens was pheromone driven, or due to malaise of the infected colony.

The head width comparison suggested that the parasite did not significantly affect polyethism within the worker caste of infected polygyne mounds. Colonies do not energetically compensate with a decrease in major worker production, even though the energetic cost of a large worker is four times that of a small worker (Calabi and Porter, 1989). The effects on polyethism could be minimized because of the decrease of worker polymorphism in polygyne RIFA (Greenberg et al., 1985), and should be investigated within the context of *T. solenopsae* infection in monogyne RIFA mounds.

Overall, the results of lower brood mass in mounds infected with *T. solenopsae*, the reduced queen output in infected colonies and the low frequencies of infected non-inseminated queens recovered in these infected colonies demonstrate some of the chronic debilitating effects of *T. solenopsae* in RIFA. These results coupled with previous research warrant continued investigation and use of *T. solenopsae* as a means to mitigate RIFA effects. The increased numbers of queens in a mound infected with *T. solenopsae* could be a response by a colony to sustain viability, or the lack of reaction by workers when presented with new reproductive members entering an infected colony.

CHAPTER IV

STABLE TEMPERATURE EFFECTS ON *Thelohania solenopsae* SPORE NUMBER AND TYPES

Introduction

Numerous questions pertaining to laboratory culturing of a pathogen must be addressed before a biological control effort can be successful on a large scale. This study addresses the need to understand the environmental parameters necessary to maintain and efficiently produce *T. solenopsae* in culture. Since *T. solenopsae* produces free spores, megaspores, and meiospores, future research may dictate the need to preferentially produce one particular type of spore over others. A controlled environment experiment was conducted to elucidate the optimum temperatures for culturing *T. solenopsae* in its host, RIFA, or temperatures at which a particular spore type is produced in worker RIFA. This study tested the hypothesis that there is no effect of different stable temperatures on the numbers or types of spores produced by *T. solenopsae*.

Materials and Methods

Fourteen RIFA colonies were collected using procedures described by Banks et al. (1981). All RIFA colonies were inspected visually with a Zeiss® phase contrast light microscope at 400× to determine whether *T. solenopsae* spores were present in workers. Six RIFA colonies infected with *T. solenopsae* and three uninfected by *T. solenopsae* were set up in each of three separate Percival® environmental chambers. Both the infected and uninfected colonies consisted of five dealated reproductive females, 2g of brood, and 2g of worker ants housed in a 12½”×6¾”×3¾” (31.75cm×17.15cm× 9.53cm)

Stacking Shoe Box. The colony nest consisted of a 100mm×15mm Falcon® Petri dish half filled with Castone and provided food *ad libitum*.

The experimental temperatures were set at 16±1.5˚C, just above the RIFA temperature for sustaining colony functions (personal observation), 26±1.5˚C, an intermediate temperature closely approximating normal room temperature within the laboratory, and 31±1.5˚C, the upper threshold temperature for RIFA viability in laboratory colonies (personal observation). Approximately 50 worker ants were collected in Eppendorf® tubes from each colony Castone® dish previous to feeding once every two weeks for 10 weeks, and frozen at -20˚C until needed. From each of these samples, 15 worker ants were selected and weighed from each sub-colony. These ants were then homogenized in 500µl of water using a Mini Bead Beater® and 0.5mm beads. An aliquot of 20µl was then examined under phase contrast light microscopy at 400×. All spore types were identified and counted per each sample. In addition, queen mortality was assessed at the times worker ants were collected. Masses of brood and surviving queens were recorded at 10 weeks, when the experiment was terminated.

The data compiled for this experiment were analyzed using a Repeated Measures General Linear Model to detect differences within and between the three experimental temperatures. Separate analyses were performed for single spores and binucleate free spores. Where warranted, post-hoc Fisher's LSD tests were performed to discriminate among means. Data were analyzed using SPSS version 12.0 (SPSS, Inc., Chicago IL, 2001).

Results

The different temperatures, 16˚C, 26˚C and 31˚C, had an effect on meiospore production ($F=7.328$, $df=2,5$, $P< 0.001$). Over the 10 week course the number of spores decreased at all temperatures ($F=11.067$, $df=5,1$, $P<0.001$) (Fig.5). There was a significant interaction between week and temperature ($F=2.714$, $df=10,1$, $P=0.004$). Production of single nucleate spores derived from meiospores decreased at all temperatures over the course of 10 weeks, but the lowest temperature of 16˚C had an apparent greater decrease (Table 1).

There was a significant effect of temperature on binucleate free spore production ($F=24.323$, $df=2$, $P<0.001$) and a significant effect over time ($F=11.917$, $df=5$, $P<0.001$). There was a significant interaction between time and temperature ($F=6.100$, $df=10$, $P<0.001$). Free spore production appeared to increase over time only at 31˚C and production of free spores appeared to decrease at 26˚C and 16˚C (Fig. 6), though the difference between the latter two was not significant (Table 2).

Table 1. Meiospore production differences at three stable temperatures. Significant at α=0.05

(I) temperature	(J) temperature	Mean Difference (I-J)	Std. Error	Sig.
16	27	-145.5933(*)	42.50012	.002
	31	-145.4153(*)	47.51658	.005
27	16	145.5933(*)	42.50012	.002
	31	.1780	47.51658	.997
31	16	145.4153(*)	47.51658	.005
	27	-.1780	47.51658	.997

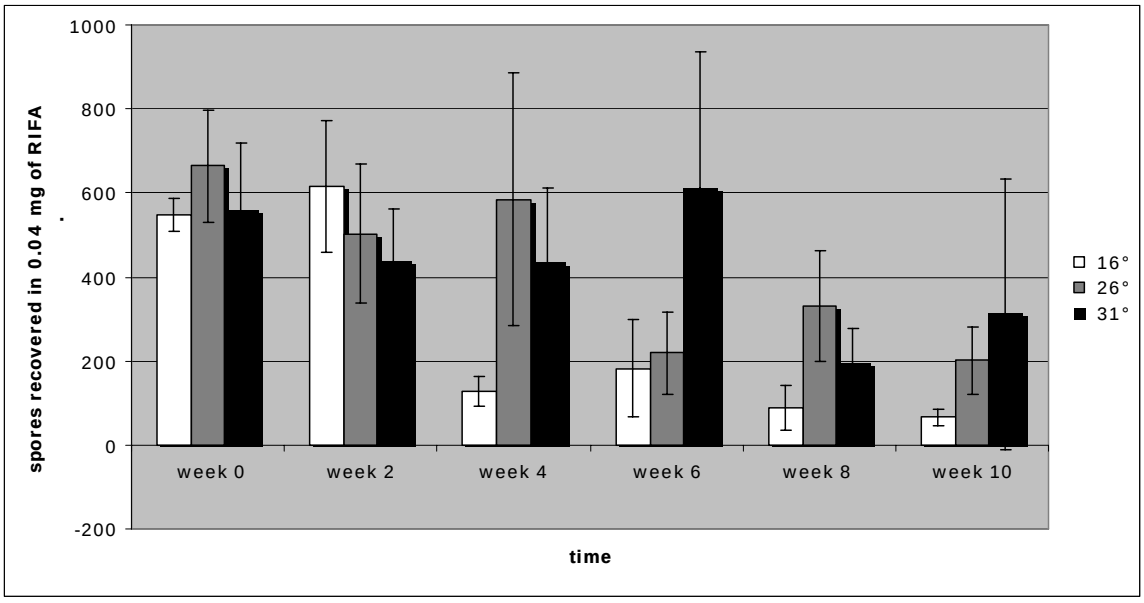

Fig. 5 Mean spores per week count in worker derived spores of RIFA

Table 2. Differences in bi- nucleate free spore production.

(I) temperature	(J) temperature	Mean Difference (I-J)	Std. Error	Sig.
16	26	-5.8237(*)	1.70000	.005
	31	-5.8166(*)	1.90066	.013
26	16	5.8237(*)	1.70000	.005
	31	.0071	1.90066	1.000
31	16	5.8166(*)	1.90066	.013
	26	-.0071	1.90066	1.000

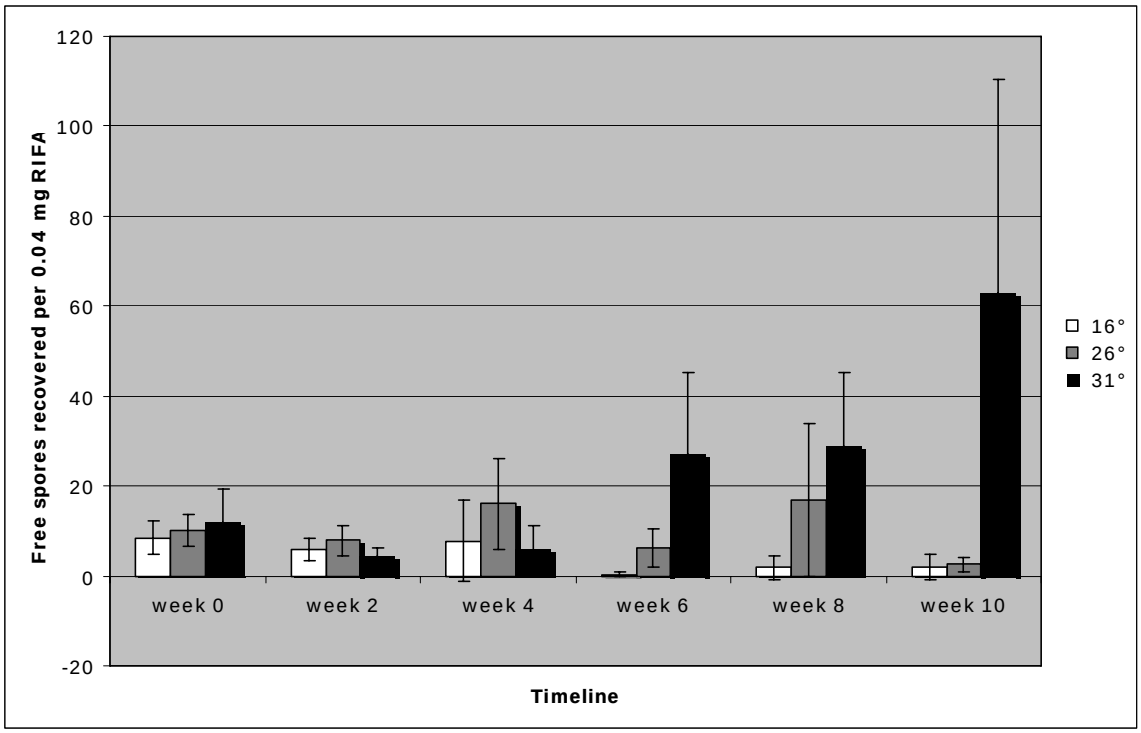

Fig. 6 Mean binucleate spores per week derived from worker RIFA

Discussion

The purpose of this study was to assess if sustained stable temperatures could affect the production and/or the type of spores produced by *T. solenopsae* in worker RIFA. The results suggested that both time and temperature affect spore production by *T. solenopsae*. The most representative spore within worker samples of RIFA were single nucleate spores.

At 16˚C, host RIFA colonies slowed all activity. RIFA workers did not feed vigorously at the honey water stations, did not use water provided in the water tubes, fed little on the crickets provided as a protein source, and did not recruit ants to a food source. Cold temperatures that affected RIFA indirectly may have affected *T. solenopsae*, since its mobility is directly dependent upon RIFA (Williams et al., 1999). The decrease of spore production may be more dramatic at this temperature, relative to the others because the horizontal and vertical transmission of *T. solenopsae* is slowed or halted There was no significant difference of single spore production between the 26˚C and the 31˚C temperatures, yet decreases in single spore production were evident at both temperatures. RIFA at these temperatures may have had enough heat input from the environment to sustain colony activity, so horizontal transmission could be maintained (Williams et al., 1999). The general decrease in spore production may be attributed to the decrease in vertical transmission, with infected queens in the study laying fewer eggs, thus producing less infected brood that may pass the infection to other larvae.

In contrast, there was a significant increase of binucleate free spore production in worker RIFA at the 31˚C. This temperature is near the upper-most temperature that RIFA can be continuously maintained in the lab, as described above, and is a close approximation of

upper-temperature thresholds for RIFA (O'Neal and Markin, 1975). Higher sustained temperatures in field conditions can be managed by RIFA through movement of brood, queens, and workers (Kuriachan and Vinson, 2000).

The increased production of binucleate free spores could be a direct response to the stress of the host, due to the potential mortality of the host source from desiccation, heat exposure (Callcott and Collins, 1996), or from the increased metabolic rate of worker RIFA.The increase of free spores in worker RIFA has a practical utility, and could be used to decrease the need for destructive sampling of reproductive RIFA. The results may also correlate to a survival mechanism in vivo when abiotic factors compromise or kill the host of *T. solenopsae;* an environmentally stable infective spore is able to survive a period without host contact.

CHAPTER V

EFFECTS OF STORAGE MATRICES AND pH VALUES ON SPORE VIABILITY DISCERNED BY CALCOFLUOR WHITE M2R AND SYTOX GREEN DUAL STAINING

Introduction

One approach to biological control of RIFA is to augment populations of *T. solenopsae* in the environment. Currently, there are no published reports on spore viability of *T. solenopsae* when removed from the host, RIFA, and none on spore viability when spores are infused or stored in oils (fats), proteins, or carbohydrates, classes of food ingested by RIFA. This study sought to answer questions concerning viability of *T. solenopsae* spores via a dual staining method, Calcoflouor White M2R followed by Sytox Green, and indirectly evaluate the potential of stored spores to infect in healthy colonies.

RIFA colonies utilize oils (fats), protein, and carbohydrates to maintain colony function and growth. These food classes are ingested by workers, then processed and moved throughout the colony (Sorensen et al., 1983). Carbohydrates are generally horded by the workers, with little sharing with nurses, or larvae. Oils (fats) are usually stored for long periods in the crops of workers, with some sharing with nurses and brood, especially reproductive brood. Ant mid-guts produce few if any proteases (Petralia et al., 1980) and mid-gut restriction prohibits large particle transit, and the buccal tube in worker RIFA filters out particles >0.88μm (Glancy et al., 1981), including the sizes of both free spores and meiospores (Knell et al., 1977). Protein synthesis is thus performed by the brood (Sorensen et al., 1983). This observation has researchers concentrating on

larval stage of RIFA which lack a buccal tube, and so allow spore passage into the midgut, initiating infection within the individual, and the colony. Since these three food classes, oils, proteins, and carbohydrates, are attractive to RIFA, they are potential bait matrices for spores. Thus, it is important to assess spore viability following storage in any of these matrices. This study tested the hypothesis that there is no difference in spore viability related to storage in oil, protein, and carbohydrate matrices over time.

There is no universal stimulant for activation of microsporidian spores. Conditions that activate spores vary widely among species, presumably reflecting specific adaptations to hosts and external environments (Undeen and Epsky, 1990). Various pH conditions have been shown to promote spore discharge, including incubation at an alkaline pH (Undeen and Avery, 1984), at an acidic pH (de Graaf et al., 1993), or even a shift in pH (Weidner et al., 1994). The efficacy of utilizing a dual staining technique of Calcoflour M2R, and Sytox Green has been demonstrated (Green et al., 2000), and this dual stain method was used in this study to measure spore viability as it relates to different pH values, and assess pH values that would be ideal for storage, or eversion of *T. solenopsae* spores. The hypothesis tested in this study was that there are no spore viability differences between discreet pH values.

Materials and Methods

Approximately 50 RIFA workers and approximately 30 individual dealated reproductives were taken from colonies assessed positive for *T. solenopsae* infection. The workers were homogenized using 0.5 mm beads in a Mini bead beater® and 500µl of water in each Eppendorf® tube. This procedure was repeated 25×, and the resulting homogenate was strained into a 500ml Falcon® tube using a Falcon® 40 micron screen

to remove larger ant debris. 200µl of this homogenate was pipetted into 100 1.5ml centrifuge tubes. Modified centrifuge techniques derived from Shapiro et al. (2003) were used to pellet the *T. solenopsae* spores. The centrifuge spun at 6000 rpm for 5 min, and the resultant pellet was re-suspended in 100µl of water and then centrifuged at 6000 rpm for an additional 5 min. Resultant pellets containing primarily single nucleate spores were either: i) re-suspended in 99µl of distilled water to check for viability ratios after centrifugation, ii) re-suspended with 99µl egg albumin solution, iii) re-suspended in 99µl 10% glucose solution, iv) re-suspended with 99µl 20% glucose solution, or v) re-suspended in 99µl of high oleic safflower oil. Spores in the various centrifuge tubes were held at 7˚C, then brought to room temperature and checked weekly for 10 weeks using the methodology described by Green et al. (2000) for discriminating between viable and dead microsporidian spores. The methodology employed dual staining Sytox Green and Calcofluor White M2R. This dual stain relies on microsporidian biology of polar filament eversion, which creates a spent spore incapable of later infecting a host cell. Because of the eversion of the polar filament, Sytox Green is allowed into the spore where it can stain the nuclear component. The Calcofluor stains a chitin component found in all microsporidian spores. This method was used to detect binucleate free spores, the only difference in methodology was that dealated reproductives replaced the workers at the first step of homogenization. All statistical analyses performed used SPSS version 12.0 (SPSS, Inc., Chicago IL, 2001). Proportions of probable viable spores were arc-sine $\sqrt{x}$ transformed. In order to test for between subject differences in food classes, a post hoc Tukey's Honestly Significant Difference (HSD) tests were used to evaluate differences in food class storage efficacy.

Methods for Staining with Sytox Green and Calcofluor White M2R

1µl of Sytox Green was added to centrifuge tubes containing 99µl of carbohydrate, oil, or water solution infused with *T. solenopsae* spores. This solution was vortexed and then incubated for 6 min. A 20µl aliquot of the resultant solution was added to a haemocytometer and counter stained with 20µl of Calcofluor White M2R, a chitin binding stain, and assessed at 400×. Yellow-green ovals evident through the 470-490 nm excitation wavelength used for Sytox Green staining were counted as dead spores. Turquoise or white ovals at 395nm to 415 nm excitation wavelength filter used for Calcofluor White M2R staining were counted as total spores. Five haemocytometer fields per slide were counted for the two centrifuge tubes in each of the four solution types each week for 10 weeks. These numbers combined gave a ratio of dead spores to total spores in the sample.

Spore Viability Detection at Different pH Values

Samples of approximately 15-30 worker ants were gathered from known infected laboratory colonies. Ants were macerated using 1.5ml Mini Bead Beater tubes, and 0.5mm beads, then samples were pooled in a Falcon® 500ml tube. This supernatant was passed through a 40 micron Falcon® filter to remove large ant debris. 400 µl of this pooled sample were poured into 1.5ml centrifuge tubes, and centrifuged using a modified Shapiro (2003) centrifugation methodology. The supernatants were then removed with a pipette, and the resulting pellets were re-suspended in 100µl of a predetermined pH solution. The pellets were allowed to remain in the pH solutions for 24 h before being stained by Calcoflour White M2R and counter stained using Sytox Green nuclear stain as described above. All spores in five haemocytometer fields were counted under a

fluorescing scope at 400×. All spores whose spore wall was compromised, and thus nuclear stained by Sytox Green, were counted. Proportions of putative living spores to dead spores were then scored. Univariate General Linear Model statistical analysis was applied to arc-sine √x transformed proportions. A post-hoc Fisher's Least Squares Difference (LSD) test was used to find significant differences among pH categories.

Results

In the queen derived spores, the viability of *T. solenopsae* decreased significantly in all food classes over time ($F=14.963$, $df=10$, $P<0.000$) (Fig. 7). There was an effect of food class on the viability of the queen-derived spores ($F=5.889$, $df=4$, $P=0.039$). There was a significant interaction between week and food class ($F=3.480$, $df=40$, $P=0.039$). The only food class treatment that differed significantly from the others was the 20% glucose concentration which showed the greatest decrease in viability over time. Other food infusions differed, but not significantly. All means and standard error are reported in the Appendix (Table A1). All results of post-hoc Tukey's HSD are reported in the Appendix (Table A2).

In the worker derived spores viability of *T. solenopsae* spores decreased in all food classes over time ($F=27.373$, $df=10$,$P<0.001$) (Fig.8). There was an effect of food class on the viability of worker derived spores ($F=9.638$, $df=4$, $P=0.014$), and a significant interaction between time and food class ($F=3.723$, $df=40$, $P<0.001$). All means and standard errors are reported in (Table 5). All results of post-hoc Tukey's HSD analysis of the between subject food class are provided in the Appendix (Table A1).

There were significant differences among spore viabilities at the different pH values ($F=13.039$, $df=11$, $P<0.001$). No spores could be detected at the acidic pH values

of 1 and 2. This nil spore viability did not significantly differ from the spore viability of pH 12 a highly basic pH. At pH 6, there was a higher viability ratio than pH 12, but is not significantly different than pH 12. The highest viability was detected at pH 4 (Fig. 9).

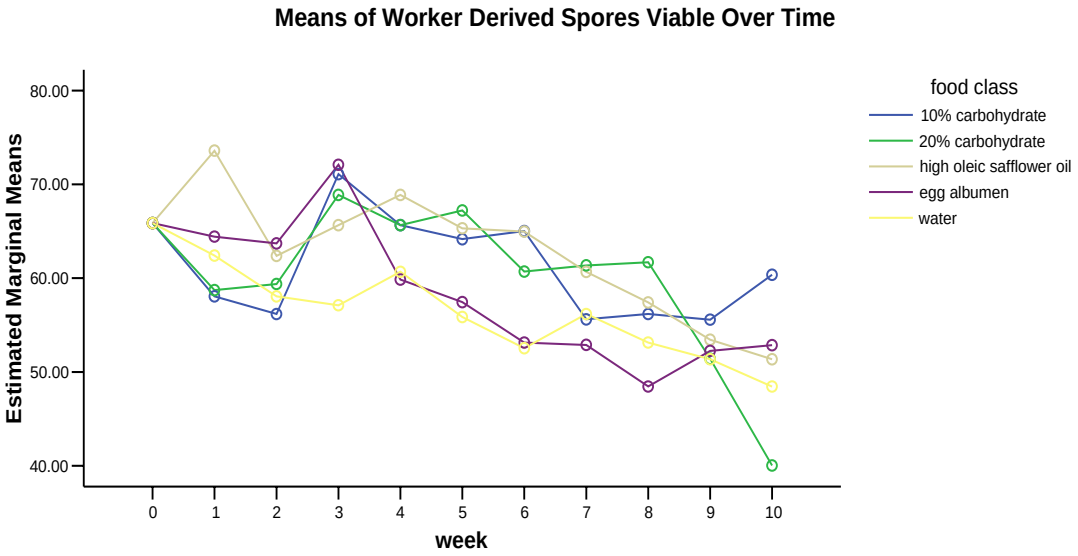

Fig. 7 Worker derived spores in various food classes viable over time

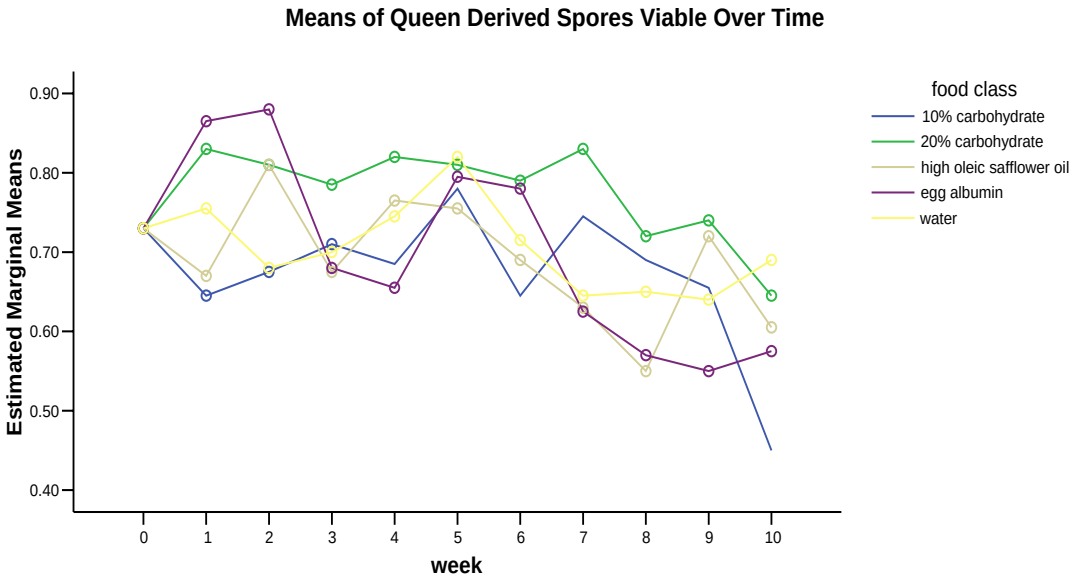

Fig. 8 Queen derived spores in various food classes viable over time

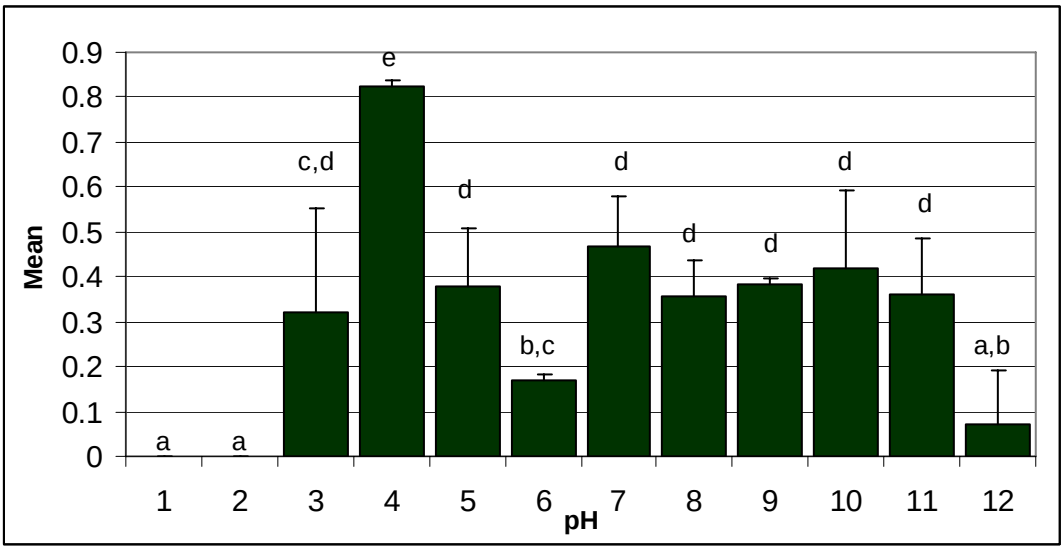

Fig. 9 Recovered viable spores after 24h. at discreet pH parameters.
Bars topped by different letters significantly different at (α=0.05)

Discussion

The goal of this study was to examine if food matrices, and pH affect the viability of *T. solenopsae* over time. The results showed that spores remained viable after centrifugation for extraction, and after infusion into a food substrate. Even after refrigerated storage for 10 weeks, these spores retained more than 40% viability. This observation is applicable for both the worker derived spores and queen derived spores. These observations suggest that unsuccessful infection efforts using food as the matrix for introduction are not due to lack of viable spores. There was a significantly greater decrease of spore viability in a supernatant of 20% glucose in comparison to 10% glucose. At 20% glucose concentration, the solution may have too much osmotic pressure, and causes more polar filament extrusion over time (Undeen, 1990, Undeen and Vander Meer 1999). Yeast growth occurred in the treatments, which may have partially obstructed visual assessment. Yeast contains a chitin component, and fluoresces using Calcofluor White M2R®. Yeast was most apparent in the egg albumen suspension; although some yeast growth was observed after 5 weeks in all storage matrices.

In the experiment examining potential effect of pH on spore viability, the lack of spores at pH 1 and pH 2 indicated that the spore wall itself was digested. At pH 12, a very basic pH, most spores were inviable after 24 h. At pH 6, which is only slightly acidic, spore viability was not significantly different than at pH 12. The highest proportion of viable spores occurred at pH 4 after a 24 h. period. The original intent was to find a stable pH that would keep the greatest proportion of *T. solenopsae* spores viable over time. However, the results of this study suggested that the spores of *T. solenopsae* may be activated by slight changes in pH. Follow up studies should address pH in the

context of RIFA physiology. Specifically, studies should address the pH parameters of the meconium, larval ant digestive secretions, and gut epithelium. These observations may correlate the observations of pH activation with processes that the *T. solenopsae* spore undergoes in order to infect RIFA.

Taken together, my results suggest that the inability to infect RIFA with ingested *T. solenopsae* spores, was not due to lack of viable spores in the food matrix, and that *T. solenopsae* spores have a shelf life in food matrices. Low pH values digest the spore wall leaving no spores to detect. A pH value of 4 keeps spores from everting after a 24 h. period and the disparity between the high viability at pH 4 and the low viability at pH 6 could be a trigger for spore eversion that emulates activation in RIFA.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The first objective of this thesis was to determine if *T. solenopsae* had any effect on polygynous colony fitness as it pertains to brood mass, queen numbers, insemination status of those queens, adoption rates and size of the workers. The results of this study showed that brood mass in mounds is lower in infected colonies, there are more queens in infected colonies, and there is not a significant difference in the worker size between infected and uninfected colonies. Within infected colonies, infected but not inseminated reproductives occurred less frequently than inseminated and infected reproductives. The higher queen numbers in *T. solenopsae* infected mounds could be a compensatory reaction in the colony to keep brood masses stable. When all effects are considered, *T. solenopsae* shows promise in biological control efforts. Colonies are known to brood raid (Tschinkel, 1992) which is surmised to be one method of inoculation in an uninfected colony (Oi et al., 2001, Williams et al., 1999). Colonies are known to adopt established intercolonial queens (Vander Meer and Porter, 2001). So, if there are more potential queens for adoption that are infected, and also inseminated, the potential for adopting a *T. solenopsae* carrier that is capable of spreading infection increases. The capability for spreading the disease exists, and may not require repeated introductions that are labor intensive, and costly.

The second objective concentrated on RIFA as an exothermic incubator of *T. solenopsae*, and the effect of temperature on spore numbers and types. Stressing RIFA with heat induces an increase of the rarer, bi-nucleate free spore type within the worker RIFA. In order to harvest *T. solenopsae* spores one must destroy the RIFA individuals

that harbor the spores, and destructive sampling of workers is more feasible than destroying the reproductives that perpetuate a colony. Using environmental chambers or greenhouse conditions to manipulate higher colonial temperatures could spare reproductives, and maintain colonies with acceptable yields of bi-nucleate free spores from worker RIFA.

The third objective focused on effects of storage matrices and pH on *T. solenopsae* spore viability. The centrifugation techniques to date produce viable spores that are not occluded by the host cell, which will further studies into the biology and physiology of *T. solenopsae*. The experiments demonstrated a decrease in viability over time, indirectly demonstrating that probable viable spores were indeed viable. The pH value of 4 retained the most viable spores after a 24 h. period. All food matrices retained viable spores through the 10 week trial. Future investigations can concentrate on how to move the spore into the colony through ingestion, and tropholaxis. If feeding experiments give positive results, the concerted efforts of spreading infections can shift away from introduction of infected brood to infect a new colony, and toward infections initiated by the usage of baits.

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APPENDIX

Table A1. Means of viable queen RIFA derived spores over a 10 week course.

Queen Derived Spores	Mean spore viability in oil	Mean spore viability in 10% carbohydrate	Mean spore viability in 20% carbohydrate	Mean spore viability in protein	Mean spore viability in water
Week 0	0.695±0.085	0.695±0.085	0.695±0.085	0.695±0.085	0.695±0.085
Week 1	0.67±0	0.645±0.025	0.83 ±0	0.865±0.025	0.755±0.005
Week 2	0.81±0	0.675±0.005	0.81 ±0.04	0.88 ±0.05	0.68 ±0.02
Week 3	0.675±0.045	0.71 ±0.04	0.795±0.005	0.68 ±0.03	0.72 ±0.02
Week 4	0.765±0.015	0.685±0.085	0.82 ±0.02	0.655±0.015	0.745±0.015
Week 5	0.755±0.035	0.78 ±0.01	0.81 ±0.02	0.795±0.015	0.82 ±0.02
Week 6	0.69±0.05	0.645±0.065	0.79 ±0.02	0.78 ±0.03	0.715±0.025
Week 7	0.63±0.05	0.745±0.035	0.83 ±0	0.625±0.045	0.645±0.015
Week 8	0.55±0.02	0.69 ±0.02	0.72 ±0.03	0.57 ±0	0.685±0.045
Week 9	0.72±0.01	0.655±0.015	0.74 ±0.01	0.55 ±0.05	0.64 ±0.02
Week 10	0.605±0.035	0.45 ±0.05	0.645±0.045	0.575±0.025	0.63 ±0.04

Table A2. Differences of viable queen derived spores in water and four food matrices.

(I) cases	(J) cases	Mean Difference (I-J)	Std. Error	Sig.
ratio of queen spores viable in 10% carbohydrate solution	ratio of queen spores viable in 20% carbohydrate solution	-6.4468(*)	1.43229	.032
	ratio of queen spores viable in oil	-1.0727	1.43229	.935
	ratio of queen spores viable in protein	-2.0136	1.43229	.650
	ratio of queen spores viable in water	-2.0032	1.43229	.654
ratio of queen spores viable in 20% carbohydrate solution	ratio of queen spores viable in 10% carbohydrate solution	6.4468(*)	1.43229	.032
	ratio of queen spores viable in oil	5.3741	1.43229	.064
	ratio of queen spores viable in protein	4.4332	1.43229	.123
	ratio of queen spores viable in water	4.4436	1.43229	.122
ratio of queen spores viable in oil	ratio of queen spores viable in 10% carbohydrate solution	1.0727	1.43229	.935
	ratio of queen spores viable in 20% carbohydrate solution	-5.3741	1.43229	.064
	ratio of queen spores viable in protein	-.9409	1.43229	.958
	ratio of queen spores viable in water	-.9305	1.43229	.959
ratio of queen spores viable in protein	ratio of queen spores viable in 10% carbohydrate solution	2.0136	1.43229	.650
	ratio of queen spores viable in 20% carbohydrate solution	-4.4332	1.43229	.123
	ratio of queen spores viable in oil	.9409	1.43229	.958
	ratio of queen spores viable in water	.0105	1.43229	1.000
ratio of queen spores viable in water	ratio of queen spores viable in 10% carbohydrate solution	2.0032	1.43229	.654
	ratio of queen spores viable in 20% carbohydrate solution	-4.4436	1.43229	.122
	ratio of queen spores viable in oil	.9305	1.43229	.959
	ratio of queen spores viable in protein	-.0105	1.43229	1.000

Table A3. Means of viable worker RIFA derived spores over a 10 week course.

Worker Derived Spores	Mean spore viability in oil	Mean spore viability in 10% carbohydrate	Mean spore viability in 20% carbohydrate	Mean spore viability in protein	Mean spore viability in water
Week 0	0.83±0.071	0.83±0.071	0.83±0.071	0.83±0.071	0.83±0.071
Week 1	0.92±0.014	0.81±0.085	0.72±0.028	0.73±0.042	0.785±0.035
Week 2	0.785±0.007	0.795±0.134	0.69±0.028	0.74±0.042	0.72±0.028
Week 3	0.83±0	0.905±0.021	0.895±0.007	0.87±0.014	0.705±0.021
Week 4	0.87±0.014	0.745±0.092	0.83±0.014	0.83±0	0.76±0.028
Week 5	0.825±0.035	0.71±0.042	0.81±0	0.85±0.014	0.685±0.007
Week 6	0.82±0.042	0.64±0.014	0.82±0.057	0.76±0.042	0.63±0.014
Week 7	0.76±0	0.635±0.078	0.68±0.071	0.77±0.028	0.69±0.028
Week 8	0.71±0.014	0.56±0	0.69±0.028	0.775±0.021	0.64±0.028
Week 9	0.645±0.035	0.625±0.035	0.68±0.042	0.61±0.085	0.61±0.028
Week 10	0.61±0.014	0.635±0.049	0.755±0.035	0.415±0.12	0.56±0.014

Table A4. Differences of viable worker derived spores in water and four food matrices.

(I) case	(J) case	Mean Difference (I-J)	Std. Error	Sig.
ratio of worker spore viable in 20% carbohydrate solution	ratio of worker spores viable in 10% carbohydrate solution	-1.1682	1.09555	.335
	ratio of worker spores viable in oil	-2.6009	1.09555	.064
	ratio of worker spores viable in protein	1.6323	1.09555	.196
	ratio of worker spores viable in water	3.5714(*)	1.09555	.022
ratio of worker spores viable in 10% carbohydrate solution	ratio of worker spore viable in 20% carbohydrate solution	1.1682	1.09555	.335
	ratio of worker spores viable in oil	-1.4327	1.09555	.248
	ratio of worker spores viable in protein	2.8005	1.09555	.051
	ratio of worker spores viable in water	4.7395(*)	1.09555	.008
ratio of worker spores viable in oil	ratio of worker spore viable in 20% carbohydrate solution	2.6009	1.09555	.064
	ratio of worker spores viable in 10% carbohydrate solution	1.4327	1.09555	.248
	ratio of worker spores viable in protein	4.2332(*)	1.09555	.012
	ratio of worker spores viable in water	6.1723(*)	1.09555	.002
ratio of worker spores viable in protein	ratio of worker spore viable in 20% carbohydrate solution	-1.6323	1.09555	.196
	ratio of worker spores viable in 10% carbohydrate solution	-2.8005	1.09555	.051
	ratio of worker spores viable in oil	-4.2332(*)	1.09555	.012
	ratio of worker spores viable in water	1.9391	1.09555	.137
ratio of worker spores viable in water	ratio of worker spore viable in 20% carbohydrate solution	-3.5714(*)	1.09555	.022
	ratio of worker spores viable in 10% carbohydrate solution	-4.7395(*)	1.09555	.008
	ratio of worker spores viable in oil	-6.1723(*)	1.09555	.002
	ratio of worker spores viable in protein	-1.9391	1.09555	.137

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