PATHOPHYSIOLOGY AND TRANSMISSION OF *Thelohania solenopsae* IN THE

RED IMPORTED FIRE ANT, *Solenopsis invicta*

A Dissertation

by

JOHNNY SHOU-CHUNG CHEN

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

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Entomology

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ABSTRACT

Pathophysiology and Transmission of *Thelohania solenopsae* in the Red Imported Fire

Ant, *Solenopsis invicta*. (August 2004)

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Thelohania solenopsae are intracellular pathogens found in the red imported fire

ant, *Solenopsis invicta*. These pathogens cause detrimental effects to their fire ant hosts.

The present study revealed that the midgut and the meconium materials from pupating

fourth instar larvae were possible vehicles for the horizontal transmission of the disease.

The pathogen was further found to cause a reduction of humeral proteins. In SDS-

PAGE stained with silver, several proteins were observed only in controls but not in

infected fire ant queens. Different queens were found to have variable proteins reduced

due to infection of this pathogen. Furthermore, vitellogenin titers were found to be

significantly reduced in infected fire ant queens, although the infection rates of the fat

body cells was found to be less than 20%. Finally, although the pathogens did not

directly induce apoptosis in infected cells, there were more infected cells undergoing

apoptosis than uninfected cells. There was no evidence to support the idea that infected

fat body cells became more resistant to apoptosis inducers.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　　Page

CHAPTER I

INTRODUCTION

1.1 The impacts of fire ants in the U. S.

The two species of imported fire ants (*Solenopsis invicta* Buren and *S. richteri* Forel) and their hybrids cause serious problems in the Southern States of the US. *S. invicta* is the most predominant of the three forms of fire ants in North America (Taber, 2000). Historically, the imported fire ants were first accidentally imported in Mobile, AL in the 1930's. Since then, due to the lack of natural enemies, high reproductive rates, and highly aggressive and opportunistic behavior, they have rapidly spread all states along the Gulf Coast. Their opportunistic behavior enabled them to act as stowaways in the soil of commercially distributed plants entering southern California (Porter and Savignano, 1990; Dowell and Krass, 1992).

Imported fire ants cause many problems. First and foremost, their highly aggressive behavior in response to nest disturbance results in painful stings to people, pets, wildlife and livestock. Approximately 0.6% of the people that are stung become hypersensitive to the stings and develop systemic anaphylactic shocks which requires immediate medical attention (Adams, 1986; de Shazo et al., 1990). Secondly, because these ants are also known to tend hemipterous pests of agriculturally valuable crops, they render biological control of these crops difficult (Vinson and Scarborough, 1991). They are also directly

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This dissertation follows the style and format of Journal of Invertebrate Pathology.

 responsible for the losses of such agricultural crops as hays and soybeans (Barr and Drees, 1996).

1.2 Chemical control strategies

The earliest methods to control the red imported fire ants were chemical controls. Two broad types of insecticides were used against these ants: contact poisons and stomach poisons. Early attempts included chlorinated hydrocarbons, such as heptachlor, which acted as contact or digestive poisons. Mirex was used primarily as a digestive insecticide against these ants. Although early control attempts were quite effective, concerns grew as the poisons were detected in non-target organisms (Taber, 2000). Another major concern of these chemicals was environmental accumulation since they failed to biodegrade, which caused their demise. Since the demise of mirex, various other types of chemicals were devised to combat the fire ants. According to Taber (2000) these chemicals fall into the following categories: delayed-action chemicals, insect growth regulators, oviposition inhibitors, inhibitors of detoxification enzymes, and inhibitors of protein digestion by the 4th instar larvae.

In the late 1990's the Texas Agricultural Extension Service (TAES) endorsed three programs to control fire ants (Taber, 2000). The first program, intended for large areas, was known as the two-step method. The first step required the broadcast of a bait, such as hydromethylnon, once or twice a year. The second step treated mounds in difficult to treat areas or those that escaped treatment in the first step. The second program, intended for smaller areas, was essentially the second step of the two-step

method. Finally, the third program involved the application of contact insecticides every month to attempt to eradicate the colonies.

1.3 Biological control strategies

       A major problem and drawback with the chemical control of fire ants was that it only worked locally while infestations remained extensive. Another major concern was the negative impact on nontarget vertebrate and invertebrate species. Chemicals may remain in the environment for years, thus biodegradability is a major concern (Taber, 2000). Therefore, current research was refocused on biological control. Various micropathogens were found to infect the red imported fire ant, including bacteria (*Pseudomonas syringae*) and three species of microsporidia (*Burenella dimorpha*, *Vairimorpha invictae*, and *Thelohania solenopsae*). *Bacillus thuringiensis* (Bt) was attempted; however, Jouvenaz et al., (1996) discovered that the food filter in the fire ants prevented the entry of the bacteria. Nematodes (*Heterorhabditis heliothidis*, *Steinernema caropcapsae*, and *Tetradonema solenopsis*) (Jouvenaz and Marin, 1992; Knutson and Drees, 1998), and fungi (*Metarhizium anisopliae, Myrmecomyces annellisae*, and *Beauveria bassiana*) (Knutson and Drees, 1998) are also known to infect the imported fire ants. The nematodes, though effective at killing the sexuals and disrupting the nest, were not very effective in the long term because after application, the fire ants left the infected nest. (Drees et al., 1992). In the case of fungal application, Oi et al. (1994) showed that *B. bassiana* reduced the foraging activities of treated nests. Also, *Metarhizium* is known to infect and kill exposed queens (Taber, 2000).

Unfortunately, the fire ants combat these pathogens using venom which was found to inhibit fungal spore growth (Storey et al., 1991).

The micropathogen that receives the most attention is *Thelohania solenopsae,* one of the three microsporidian species. They were first discovered in alcohol-preserved specimens from Brazil (Knell et al., 1977). They were not discovered in North America until 1998 (Williams et al., 1998). Microsporidiosis has been suggested as an alternative biological control method for several reasons. These pathogens can cause significant decline in both laboratory and field colonies of fire ants (Briano and Williams, 1997; Williams et al., 1999). Briano et al. (1995a) found that mound densities of infected plots decreased significantly when compared to control plots. Briano and Williams (1997) found that, in starvation experiments, infected ants have significantly reduced longevity than their uninfected counterparts. Finally, infected queens have significantly lower oviposition rates than healthy queens (Williams et al., 1999). In laboratory experiments, Williams et al. (1999) found that the artificial infection of colonies caused significant decline of brood production over the course of one year. In combination with diatomaceous earth, Brinkman and Gardner (2001) found increased effectiveness than when using the pathogen alone. Therefore, Briano et al. (1995b)'s suggestion that these parasites may be a potential biological control agent against the fire ants were further supported by these findings. However, because the infection is chronic, results would not be achieved until at least three months (Williams et al., 1997; Williams et al., 1999).

A related issue that also requires attention is host specificity. In order to successfully implement *T. solenopsae* as a biological control strategy, host specificity of

the pathogen must be evaluated. In the case of *T. solenopsae* in North America, it

should be determined whether this parasite could be found in native fire ants such as *S.*

xyloni, *S. geminata*, and their hybrids. To investigate this, Briano et al., (2002) studied

the field host specificity of *T. solenopsae* in South America. They found the presence of

T. solenopsae in *S. macdonaughi*, the third species of *Solenopsis* sp. to be diagnosed

with this pathogen. They were unable to find *T. solenopae* in other genera of ants

surveyed, including *Pheidole*, *Camponotus*, *Crematogaster*, *Linepithema*,

Brachymyrmex, *Paratrechina*, *Dorymyrmex*, and *Wasmannia*.

1.4 General life cycle of *Thelohania solenopsae*

The general life cycle of *T. solenopsae*, which includes the different life stages,

was first described by Knell et al. (1977). The infection is initiated in the midgut

epithelium after the oral ingestion of spores. They found that *T. solenopsae* infections

are localized in the fat bodies of adult workers. In the case of queens, the infections are

found in the fat bodies and ovaries, which may cause infections in her progeny.

Sporonts are only found in adults and there are at least two or more documented types of

spores, the octospores and the binucleate free spores (Knell et al., 1977; Sokolova and

Fuxa, 2001). The octospores have only one nucleus per spore and are enclosed by a

pansporoblast membrane, while the free spores are not bounded by such membrane.

Also, the free spores tend to be more ovoid in outline than octospores, which are more

pyriform in appearance.   Both octospores and the free spores are found in the fat body

cells. Free spores measure 1.85+/- 0.16 X 4.93 +/- 0.58 um while octospores measure

1.95 +/-0.20 X 3.32 +/- 0.48 um.in size (Knell et al., 1977) Schizonts, on the other hand,

are only found in the larvae. Knell et al. (1977) also noted that sporulation in adults is very rapid, such that they were unable to document the earliest sporulation stages. More recently, more spore forms have been documented (Sokolova and Fuxa, 2001; Sokolova et al., 2003; 2004). These authors found "megaspores", in addition to the already recognized octospores and the free spores, and were able to verify their identity by using PCR (Sokolova et al., 2003, 2004), . Oi et al. (2001) documented, for the first time, the presence of spores in pupae.

The infection of the host by microsporidia to is thought to be initiated in the midgut epithelium (Boucias and Pendland, 1999). As in other species of microsporidia, the polar filament extrudes from the spore to allow the infective sporoplasm to enter the host cell (Tanada and Kaya, 1993). However, in species such as the imported fire ants, the mechanism by which the spores proliferate from the midgut epithelium to other tissues remains unclear. Because many microsporidia genera, such as *Nosema*, *Edhazardia*, *Thelohania*, *Vairimorpha*, and *Amblyospora* have distinct spore forms, Boucias and Pendland suggest, based on the data of Avery and Anthony (1983), that some of these spore forms may be used as in vivo stages to proliferate within the host. The meront, or the vegetative stage, may also be involved in the proliferation of the disease. According to Boucias and Pendland (1999), sporoplasms or meronts can survive in circulating hemocytes. The infected hemocytes may become sticky to aid the transfer of the developing meronts when the hemocytes bind with other tissues. Finally, the source of these vegetative stages may be from other infected tissues, such as the midgut, which may attract hemocytes. As these hemocytes ingest the infected tissues via

phagocytosis, they may become infected with the vegetative stages of the pathogens

(Boucias and Pendland, 1999).

1.5 Molecular analysis of microsporidia

Molecular analysis is an important aspect of microsporidia research because this

may help to explain the relationship of this mysterious group of organisms to other

groups. Analyses may also help to define taxonomic groups at the genus, species, and

subspecies levels. Early attempts, before the advent of molecular phylogeny and mainly

based on morphological and ecological characteristics, placed microsporidia among

protozoans. Also, Vossbrinck et al. (1987) proposed that microsporidia were ancient

protozoans due to the lack of mitochondria, and, based on comparative rDNA data, the

high degree of divergence between the microsporidia, *Vairimorpha necatrix* and other

eukaryotes.

However, more recent data suggest that they are actually highly evolved fungi.

For example, though lacking mitochondria, Germot et al. (1997) found the presence of

mitochondrial HSP-70 DNA in the genome of the microsporidia *Nosema locustae*. This

finding suggests that microsporidia may have once have possessed this organelle but

have subsequently lost it and that instead of being ancient organisms, microsporidia may

be highly derived. Comparisons of the sequences of other proteins such as β–tubulin,

suggest that microsporidia may be more closely related to fungi than they are to protists

(Edlind et al., 1994; Katiyar et al., 1994; 1995; Li et al., 1996; Weiss and Vossbrinck,

1999). Hirt et al. (1999), showed that microsporidia are more related to fungi than

protists by comparing the genetic sequences of the large subunit of RNA polymerase II

and other proteins from two microsporidia species, *V. necatrix* and *N. locustae*. Katinka et al. (2001) completed the genome sequencing of *Encephalitozoon cuniculi*. By comparing the sequences of various genes, including β-tubulin, they found that *E. cuniculi* is more closely related to fungi than protozoans.

In addition to answering questions regarding the origins of these organisms, molecular approaches are important to classify microsporidia when traditional classification based on life cycle and ultrastructure is problematic and time consuming (Baker et al., 1995). Molecular techniques determining the sequences of the ribosomal gene have become a useful approach to the problem of microsporidia species identification (Baker et al. 1994, 1995, 1997). Using this approach, Moser (1995) and Moser et al. (1998), Valles et al. (2002) and Snowden et al. (2002) were able to determine the taxonomic status of microsporidia infecting the imported fire ants in North America. In South America, it is well known that there are three major species of microsporidia that infect fire ants: *T. solenopsae*, *V. invictae*, and *B.dimorpha*. In North America, however, only *T. solenopsae* and *Thelohania* sp. have been identified thus far (Williams et al., 1998; Moser et al., 1998; 2000).

Ever since its discovery, most of the research on this pathogen has been focused on the fire ants from South America, while little has been performed with the North American counterparts, because this pathogen was not discovered there until 1998 (Williams et al., 1998). In that study, they discovered microsporidia from locations near Gainsville, FL. After comparisons with the ribosomal DNA sequences of *T. solenopsae* from Moser et al. (1998), they concluded that they found the same *Thelohania*

solenopsae from Florida as Moser et al. (1998) did from Brazilian populations of fire ants. In a follow up study, Moser et al. (2000) compared isolates of *Thelohania* from Brazil, Argentina, and North America. They failed to find any morphological differences based on ultrastructural study via transmission electron microscopy. Based on comparisons of ribosomal DNA sequences, they concluded that the North American isolates have mirror sequence variations from the South American isolates. Recently, Cook (pers. comm.) found more than one morphological forms of microsporidia infecting North American fire ant colonies. It is therefore possible that another species of microsporidia exist here, in addition to *V. invictae* and *T. solenopsae*. Thus, the taxonomic status of *Thelohania* infecting fire ants remains unclear in North America.

In other insects, host specificity has been shown to be dependent on the species of microsporidia. *Nosema locustae*, the only Environmental Protection Agency (EPA)– registered biological insecticide, has been shown to be specific for grasshoppers. Attempts to inoculate other insects such as honey bees, lepidopterans such as *Heliothis zea* and *Agrotis ipsilon* have not been successful (Brooks, 1988). This pathogen has also been shown to be safe to various species of vertebrates including mammals, fish, and birds. *N. algerae*, on the other hand, has a very broad host range. In addition to its native mosquito host (Sprague and Becnel, 1999), this parasite has been shown to artificially infect 6 different orders of insects, a crustacean (Undeen and Maddox, 1973), and the laboratory mouse (Undeen and Alger, 1976). Orally, this pathogen can infect four different families of insects and two trematodes (Brooks, 1988). Humans were also found to be susceptible to this pathogen (Cali et al., 1996; Visvesvara et al., 1999).

1.6 Possible means of transmission

Transmission is the transfer of pathogens from one host to another. There are two general means of transmission, horizontal and vertical. Horizontal transmission deals with the transmission of a pathogen within a generation of host. In entomogenous microsporidia, horizontal transmission may be achieved in various ways. Pathogens may be transmitted by oral ingestion of materials contaminated with spores. An example may include transmission of *N. apis*. Worker honey bees become infected with *N. apis* by ingesting spores in the nest while cleaning it (Becnel and Andreadis, 1999). Infected hosts may also become moribund and die and new hosts may become infected by cannibalism on the infected cadavers (Brooks, 1988). *Nosema locustae* is known to achieve horizontal transmission in the rangeland grasshoppers this way, in addition to consuming infected fecal materials (Boucias and Pandland, 1999).

Horizontal transmission may also involve an intermediate host. For example, the transmission of *Amblyospora connecticus* in *Aedes cantator* requires the copepod *Acanthocyclops vernalis* as the intermediate host (Becnel and Andreadis, 1999).

Finally, horizontal transmission may be achieved via the aid of vectors, in which the spores are mechanically transmitted by the vectors. Transmission of *B. dimorpha* is achieved in this manner (Jouvenaz and Hazard, 1978). In this case, infected larvae are cannibalized by adult workers. However, the cannibalized larvae are not ingested. Instead, the food particulates and the spores are stored in the infrabuccal cavity, where the food and the spores are fed back to the fourth instar larvae.

Vertical transmission, on the other hand, deals with the transmission of a pathogen from one generation of host to the next. In entomogenous microsporidia, this may occur via several different mechanisms. Transovarial transmission occurs when the pathogen infects the eggs via the ovary of the host. According to Becnel and Andreadis (1999), this method is the most common method of vertical transmission for entomogenous microsporidia, although the mechanism of microsporidia's entry into the egg from the ovaries and the associated reproductive structures remain unclear. Several mechanisms have been proposed. In polymorphic microsporidia, transovarial transmission occurs by direct inoculation of individual oocytes from special spores. Such examples include *Amblyospora*, *Culicospora*, and *Edhazardia* (Becnel and Andreadis, 1999; Andreas and Hall; 1979a; Andreadis, 1987; Becnel et al., 1987; 1989; Becnel, 1994).

In the case of monomorphic microsporidia, no special spore types are produced for transovarial transmission; instead, during oogenesis, the different life stages (vegetative or spore) may be incorporated into the developing eggs. Examples may include various species of *Nosema*, which are found in coleopteran, lepidopteran, and hymenopteran species (Becnel and Andreadis, 1999).

Another form of vertical transmission is the transovum transmission, which occurs when eggs become contaminated with spores from fecal materials, anal hairs, and ovarian connective tissue. The hatched larvae become infected with spores when they ingest the contaminated eggshell (Boucias and Pendland, 1999). This means of

transmission has been documented in *Nosema algerae* infection of *Anopheles stephensi* (Alger and Undeen, 1970; Canning and Hulls, 1970).

Though modes of infection and transmission of this microsporidia have been studied in fire ants (Jouvenaz and Hazard, 1978) the mechanism of horizontal transmission of the disease remains unclear. (Oi et al., 2001) found that brood brood infective to the colony. Although workers were found to aid the spread of the pathogen, the means of transmission remains unknown (Oi et al., 2001). The discovery of anal fluid produced by fourth instar larvae (Tschinkel 1995) and the presence of spores in larvae (Oi et al., 2001) may provide a possible explanation. The anal fluid is produced by fourth instar larvae and is either ingested by workers or is given by workers to other members of the colony, including the queens. If it is possible for the spores to localize in anal fluid, the fluid may be the means of horizontal transmission. This proposed method of transmission is different from the horizontal transmission of *Burenella dimorpha*, described by Jouvenaz and Hazard (1978). This organism produces two types of spores, namely the membrane-bound binucleate octospores retained in the pupae and the non-membrane-bound uninucleate free spores that are shed in the meconium. The octospores accumulate in the proventriculus of the nurse workers after they cannibalize infected larvae. The infected nurse workers, in turn, feed other workers and larvae with the infected pellet.

1.7 Pathophysiological effects of microsporidiosis

Infection by pathogens adversely alters the physiological state of the host. In entomogenous microsporidia, most of the effects have been documented for the cells

they directly infect, while there is limited research on the effects on hosts' physiology (Weidner et al., 1999). In the case of the effects on the host's immune responses, once the spores successfully infect the host cells, unless the integrity of the infected cells were compromised, the immune system is not triggered (Becnel and Andreadis, 1999). Vavra and Undeen (1970) suggested that spores, when released into the hemocoel, may be encapsulated. It has been shown that microsporidiosis of *N. grilli* causes differences in protein patterns in the hemolymph and fat bodies of crickets (Seleznev et al., 1996). In honeybees, the infection of *N. apis* causes the disintegration and vacuolation of the cytoplasm of the hypopharyngeal glands (Hassanein, 1952b). This parasite also suppresses RNA synthesis in the honey bee; however RNA synthesis is restored when treated with the drug fumagillin, a drug used to control microsporidian infection (Hartwig and Przelecka, 1971).

It is already known that *T. solenopsae* infects the fat bodies and the ovaries of fire ant queens. It is also known that vitellogenin is a necessary ingredient for egg production and that vitellogenin is synthesized by the fat body (Keeley, 1985; Lewis et al., 2001). With the infection of fat bodies by *T. solenopsae*, it is possible that infected queens may not be able to produce vitellogenin at the same rate as their healthy counterparts, leading to lowered vitellogenin titers in the hemolymph. This may in part explain the observation that infected queens have a lowered fecundity compared to uninfected queens (Williams et al., 1999).

Various types of humoral proteins exist in the hemolymph. They may include storage proteins such as hexamerins and arylphorins, lipid transport proteins such as

apolipophorins, enzymes such as trehalases and phenoloxidases, and protease inhibitors (Kanost et al., 1990; Chapman, 1998). Arylphorines are known to be synthesized by the fat body tissues. These proteins are important because they provide the raw materials for the tissues during the next molt (Chapman, 1998). Trehalases are important to the insects' energy metabolism while phenoloxidases are critical to the immune system of the insect (Tanada and Kaya, 1993; Boucias and Pendland, 1999). Apolipophorins are an integral part of the insect's neuroendocrine system because they are responsible for carrying the water-insoluble lipid hormones. Finally, protease inhibitors are important because they prevent proteins such as the pro-phenoloxidase from activation when they are not needed (Chapman, 1998).

One of the important aspects of invasions by foreign organisms includes the immune response. One of the most important aspects of the humoral response includes the pro-phenoloxidase cascades, which recognize foreign invaders and lead to melanization and encapsulation of the pathogens (reviewed by Boucias and Pendland, 1999).

1.8 Cellular pathology and apoptosis

Issi (1986) documented the cellular pathology of microsporidiosis in insects. Initially, during merogony, the host cell is stimulated to concentrate endoplasmic reticulum around the pathogen. Also, the number of mitochondria is increased and nucleic acid synthesis is stimulated. During early sporogony, the amount of endoplasmic reticulum is reduced while the vacuolation of the cell is increased (Tanabe, 1971; Liu and Davis, 1972; Issi, 1986). Later in sporogony, only the nucleus and the mitochondria remain in the host cells. In sciarid flies, for example, Jurand et al., (1967)

noted marked changes in the ultrastructure of the salivary gland cells. The microsporidial parasites deplete the ribosomes, disarrange endoplasmic reticulum, increase the number but decrease the size of mitochondria, and cause the formation of vacuoles in the host cytoplasm. In some cases, the nucleus and the chromosomes may undergo hypertrophy (Diaz and Pavan, 1965; Jurand et al., 1967; Roberts et al., 1967; Martins and Perondini, 1977). In honeybees infected with *Nosema apis*, de Graaf et al. (1994 a and b), noted that the spores will eventually fill and rupture the host cells in the midgut epithelium. In fire ants, the types of association between the parasite and the host cells remain unclear.

A related issue to the cellular pathology due to microsporidia infection is whether this pathogen may lead infected cells to undergo apoptosis. Apoptosis is one of the two means of cell death, the other one being necrosis. Whereas necrosis involves uncontrolled swelling bursting of the cells and the leakage of cytoplasmic contents, apoptosis involves cell shrinkage, phosphotidylserine externalization, cytoplasmic collapse and condensation, dissemblement of nuclear membrane, fragmentation of DNA, and the engulfment of the cells by macrophages. Scanlon et al. (1997) suggested that *N. algerae* sp. causes neither apoptosis nor necrosis. Instead, infected cells, they found became resistant to apoptotic inducers.

Apoptosis is a very tightly regulated process. Several major genes and pathways have been discovered that mediate cell death. Much of the early and current investigations on the genes and genetic pathways that regulate apoptosis have been on mammalian and nematode systems (Hengartner, 2000) with evolutionary conservation in

genes and mechanisms being found in insects (Abrams, 1999; Huang et al., 2000; Zhang et al., 2000), suggesting that genetic regulation of apoptosis is highly conserved and that investigations of mammalian aspects of apoptosis may have important implications to apoptosis in insects cells.   The most important enzymes that regulate apoptosis are known as caspases (Enari et al., 1998; Hengartner, 2000; Degterev et al., 2003). Caspases, or cysteine, aspartate specific proteases, play a central and critical role in the regulation of cell death (Hengartner, 2000). Functionally, the two general types of caspases include upstream initiator caspases, which include caspases-1, -2, 4, -5, 8, -9, -10, -11, and -12). These proteases are characterized by having long prodomains that have one of two protein-protein interaction motifs, which are death effector domains (DED) (caspases-8 and -10), and caspase activation and recruitment domains (CARD) (caspases-1, -2, 4, -5, -9, -11, and -12). The other types of caspases, which are in the downstream steps, are known as the "executioner" caspases. These proteases, which include caspases -3, -6, and -7, are characterized by having a short prodomain.

Cells utilize various means to activate these enzymes (Hengartner, 2000). Caspases may be activated by upstream caspases in a "caspase cascade" (Hentgartner, 2000). Examples of cysteine proteases that behave this way include caspases-3, -6, and -7. Another way these enzymes may be activated is through "induced proximity." Death receptors such as CD95 play a key role in this scenario. When bound with a ligand, these receptors aggregate and membrane-bound signaling complexes are formed, which, via adaptor proteins, recruit molecules of caspase-8. The resulting high levels of zymogen, which is an inactive precursor of proteolytic enzymes, allow the proenzymes

to begin cleaving one another, resulting in caspase activation. Finally, a very complex activation mechanism occurs when caspases are associated with a regulatory subunit, which is the mechanism of activation of caspase-9. The activation of these enzymes involves the formation of a holoenzyme, which is a complete enzyme complex, from cytochrome c, and ATP-dependent oligomerization of Apaf-1 and caspase-9. A conformational change of this holoenzyme activates caspase-9.

Bcl-2 family of proteins is one of the major genes discovered that regulates apoptosis (Adams and Cory, 1998; Cory and Adams, 2002). There are three types of *Bcl*-2 proteins. One type, which includes such examples as *Bcl*-x$_l$ and *Bcl*-w, is known to inhibit apoptosis, even in the presence of many apoptotic inducers. There are two models to explain how these proteins ensure the survival of the cell. In *C. elegans*, CED-9, the homologue of Bcl-2, prevents CED-4 from activating caspase CED-3, by binding to CED-4. Apoptosis occurs when BH-3 (short for *Bcl* homologous region 3) only EGL-1 binds to CED-9, displacing CED-4. In the mammalian system, the mitochondrial membrane integrity is protected by *Bcl*-2. BH-3 only proteins are used to neutralize the Bcl-2 to induce apoptosis. Cytochrome c is released when the second type of *Bcl*-proteins, which includes Bax and Bak, forms oligomers with the mitochondrial membrane. The released cytochrome c then binds with Apaf1 and caspase 9 to initiate apoptosis (Green and Reed, 1998).

Insect apoptosis has been investigated mostly in *Drosophila melanogaster* (Abrams, 1999). The mechanisms of apoptosis in insects have been found to be highly conserved (Abrams, 1999; Zhang et al., 2000; Huang et al., 2000). Insect homologues

of caspases (Abrams et al., 1999), *Bcl*-2 (Zhang et al., 2000), Apaf-1/Ced-4, (Rodriguez

et al., 1999), and IAP's (Hay et al., 1995; Huang et al., 2000) have been found. Huang

et al. (2000) showed that lepidopteran IAP's (or inhibitor of apoptosis proteins may also

inhibit mammalian caspase-9, suggesting evolutionary conservation of apoptotic

mechanisms. The activators of apoptosis in *Drosophila* are located in the *Reaper* region,

which is the first apoptotic gene identified in this insect (Lohmann, 2003). The two

other genes in this region, *Hid* and *Grim*, function with *Reaper* as important activators of

apoptosis (Lohmann, 2003). The exact mechanisms of caspase activation by these genes

remain uncertain (Abrams, 1999).

1.9 Investigations of this dissertation

This dissertation will investigate three major areas of *T. solenopsae* infection of

the red imported fire ants: possible routes of horizontal transmission of the disease,

parasite effects on humoral proteins of hosts, and possible apoptotic effects of the

pathogen on the host cells.

The first part of this dissertation, based on the results of Oi et al. (2001),

Tschinkel (1995), and Cassill and Vinson (unpublished observations), will investigate

the possible sources of spores of *T. solenopsae* involved in the horizontal transmission.

The research here will provide more evidence as to the mechanism by which the spores

may be horizontally transmitted in a colony. Currently, *B. dimorpha* remains the only

microsporidian to have the means of horizontal transmission understood. An

understanding of the manner of horizontal transmission of *T. solenopsae* may serve as a

stepping stone to further enhance our basic understanding of the life cycle of this important pathogen of the imported fire ant.

The second part of this dissertation focuses on the effects of *T. solenopsae* on the hemolymph proteins of the hosts. So far, the only similar work was by Selenev et al. (1996), who showed differences in humoral proteins in crickets infected with the microsporidia, *N. grylli*. The work here is the only other work known to deal with entomogenous microsporidia in this way. Because there are various castes in fire ants, this work may also provide additional information on any differences in humoral responses in between different castes.

The objective of the third part of the dissertation is to investigate whether or not apoptosis occurs in infected and uninfected fire ants. Because *T. solenopsae* infects fat body cells, this part of the project will focus on the fat body cells, or trophocytes. Tissues from both infected and uninfected queens will be removed from the fire ants and examined for signs of apoptosis. Also, activities of infected cells will be examined. In addition, both uninfected and infected tissues will be incubated in the presence of an apoptosis inducer to induce apoptosis. They will be compared to determine if any differences exist in the percentage of apoptotic cells. The infected cells will also be examined in this incubation experiment. These experiments are important because they are one of the first investigations of apoptosis in fire ants.

CHAPTER II

SOURCES OF SPORES FOR THE POSSIBLE HORIZONTAL TRANSMISSION OF

Thelohania solenopsae (MICROSPORA: THELOHANIIDAE) IN THE RED

IMPORTED FIRE ANT, *Solenopsis invicta**

2.1 Introduction

Imported fire ants, *Solenopsis invicta* Buren, were accidentally introduced into

North America in the 1940's (Lofgren, 1986). Due to the lack of natural enemies, high

reproductive rates, and highly aggressive and opportunistic behavior, they have rapidly

spread and colonized all of the states along the Gulf Coast and recently California

(Dowell and Krass, 1992). Because of their aggressive behavior, which results in

painful stings to people, pets, wildlife and livestock following nest disturbance, fire ants

are serious medical and economic pests (Taber, 2000).

Management in the past relied on locally applied chemical control agents;

however, infestations remain extensive (Drees et al., 1996; 1998). As a result, current

management and research efforts have been refocused on biological control strategies

(Drees and Lennon, 1998). Potential biological control agents for imported fire ants

include a species of micropathogens in the phylum Microspora. Of the 1,200 described

species in this phylum, the most common species

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infecting the imported fire ant is *Thelohania solenopsae* (Briano et al., 1995a). This pathogen was first discovered from infected, alcohol-preserved ant specimens from Brazil (Allen and Buren, 1974) and North America in 1998 (Williams et al., 1998). *T. solenopsae* is known to cause a significant decline in both laboratory and field colonies (Briano et al., 1995c; Williams et al., 1999). Briano and Williams (1997) and Briano et al. (1995b) have suggested that these parasites may serve as a potential biological control agent against fire ants.

The various life stages of *T. solenopsae* were reported by Knell et al. (1977). They described the two basic spore forms of *T. solenopsae*, which include the binucleate free spores and the uninucleate octospores. Free spores are usually larger in size and exist individually; while the octospores are usually smaller in groups of eight, enclosed by a pansporoblastic membrane (Knell et al., 1977). While it is clear that vertical transmission occurs (Oi et al., 2001), the routes of horizontal transmission remain enigmatic. These authors found that the infected brood alone was insufficient to infect uninfected queens (Williams et al., 1999; Oi et al., 2001). In other words, none of the uninfected queens became infected with only infected brood contact. However, if uninfected workers were added to experimental colonies with infected brood and uninfected queens, then a majority of the queens became infected. They also found that a small percentage of queens became infected if the uninfected queens were to be mixed with infected workers without any brood. The presence of workers is necessary as intermediates to infect the queen. Oi et al. (2001) suggested that secretions from the infected brood must be transferred by the workers to the colonies. Thus, in this report,

materials released from larvae and workers were examined for possible sources of spore

contamination. Ant middens were also examined because the fire ants pile their dead and

waste materials in piles outside of the nests (Hölldobler and Wilson, 1990, pp. 296-297).

2.2 Materials and methods

2.2.1 Ants and rearing

Ants were collected from several sites in Brazos County, TX by transferring as

much of the nest as possible into buckets using a shovel. The nests were brought to the

laboratory, held for 48 hr, and separated from soil by a drip-flotation method (Jouvenaz

et al., 1977). Essentially, the buckets containing the fire ants and the nest materials were

placed under a faucet under very slow dripping of water for several hours. As the water

level rose, the ants and the brood began floating on top of water. When the water level

in the bucket rose above the nest soil, the ants were then transferred into their laboratory

colonies in the sweater boxes. Ants were confirmed to be polygyne by the method of

Greenberg et al. (1985) by comparing the average size of head capsules because

monogyne colonies have lager workers than polygyne colonies. Colonies were

maintained and reared using previously described methods (Chen and Vinson, 1999).

Briefly, ants were reared in plastic sweater boxes (31.75 cm X 17.15cm X 9.5cm) with

the inner wall coated with Fluon® to prevent escape. Nests were made by filling 145

mm diameter Petri-dishes with castone. Two holes were drilled on top of the lid to allow

the entrance of ants into the nest. Test-tubes filled half way full with water and plugged

with cotton were also provided as nests.   The ants were fed daily with meal worms,

frozen crickets, and honey water. Ants were screened for the presence of spores by wet

mount, modified trichrome staining, and calcofluor white M2R staining (Didier et al., 1995). To prepare wet mounts and the biological stains, 0.5 g of workers were macerated in 500 μl of tris-buffered saline (TBS, pH 7.2) and 20 μl aliquots of the homogenate were used in wet mount observation of spores. The wet mounts were covered by a cover slip. The entire slide was scanned and the number of spores in the slides was counted. Only the colonies with at least 20 clearly visible spores in the wet mount slides, deemed heavily infected, were used in all subsequent assays. To ensure that the spores were that of microsporidia 60 μl of the homogenate was stained by modified trichrome and calcofluor stains. Thirty microliters of the homogenate was used in trichrome staining, after the homogenate was spotted onto a glass slide, the slide was allowed to dry overnight. In trichrome staining, the dried slide was fixed in methanol for 5 minutes. The slide was then stained in chromotrope (chromotrope 2R: 6.0 g; fast green: 0.15 g; phosphotungstic acid: 0.7g; glacial acetic acid: 3 ml; 100 ml ddH_2O) (Chemicals from Sigma) for 90 minutes. The slides were than rinsed with acid alcohol (4.5 ml acetic acid, 995 ml 90% ethanol) for 10 seconds, and rinsed briefly in 95% ethanol. The slides were then dehydrated in 95% ethanol for 5 minutes, 100% ethanol for 10 minutes, and finally, Hemo-De, a xylene substitute, for 10 minutes. In the case of calcofluour M2R staining, the methanol-fixed samples were treated with Calcofluor M2R (Sigma) for 3 minutes. The slides were then rinsed under running tap water and counterstained with 0.1% Evan's Blue (Sigma) for 1 min. The slides were then rinsed with ddH_2O and allowed to air dry. These slides were viewed under the fluorescent microscopy a Nikon Laboshot 2 microscope equipped with Nikon EFD-3 flourescent filters at the

magnification of 400X and at the excitation wavelength of 350nm and emission wavelength of 470nm.

The presence or absence of *T. solenopsae* was further confirmed in both spore positive and negative colonies using PCR amplification of a portion of the ribosomal RNA genes as previously described (Snowden et al., 2002). Briefly, approximately 50 to 300 mg of workers were homogenized in 1 ml Tris-Buffered Saline (TBS), pH 7.4 with 750 mg of disposable glass beads using a mini bead-beater (Mini-beadbeater Type BX-4 cell disrupter, Biospec Products, Bartlesville, OK), for 15 seconds at 5000 rpm. Homogenate was spotted onto a Whatman FTA card to allow to dry overnight. Two millimeter samples of the dried FTA card were then punched and placed into 1.5 ml microtubes. The samples were then washed with FTA purification reagent and with TE buffer (10mM tris + 1mM EDTA, pH 8.0, Sigma, St. Louis, MO) according to manufacturer's instructions. Only colonies confirmed to be *T. solenopsae* free were used as controls (N=13) and only colonies containing spores that were confirmed to be *T. solenopsae* by PCR were used as infected colonies (N=24).

2.2.2 Collection of adult secretions

The primary purpose of this study was to determine possible contamination of adult and larval secretions with *Thelohania* spores that may be associated in horizontal transmission. Vertical transmission has been demonstrated (Oi et al., 2001) and transmission from infected larvae to uninfected colonies occurs only if uninfected workers are present, although infected workers alone may introduce the disease (Oi et al., 2001).

The two possible sources of spore contaminated secretions from adults include regurgitated oral fluids and anal fluids (referred to here as fecal fluids). However, we found that it was not possible to collect enough salivary secretions directly from adults. Thus, crop contents were collected and pooled from 15 "replete-like" workers, based on a modification of Tennant and Porter (1991). Using a pair of sharp surgical scissors, each worker was cut in half between the two petioles. The head and the thorax were discarded. Forceps were used to depress the venter of the gaster and crop fluid was collected in 5 μl microcapillary tubes (Alltech Associates, Inc., Deerfield Il). The pooled crop fluid from the ants was diluted into 20 μl of tris-buffered saline (TBS), 10 μl aliquots were stained with trichrome, and viewed under light microscopy at 400X (Table 1). The used gasters were collected and checked for the presence of spores by trichrome staining.

Because hemolymph could have contaminated these crop samples, hemolymph was also examined for spores. Hemolymph samples of 15 replete-like workers were pooled per colony and were collected based on the protocols of Lewis et al. (2001). Briefly, each worker was fixed onto a wax block using a staple with dorsum facing the observer. A thin insect pin was used to pull the gaster until the intersegmental membranes were exposed. Another insect pin was then used to carefully drill a hole onto the intersegmental membrane. A 0.5 μl capillary tube (Alltech Associates, Inc., Deerfield, Il), was subsequently used to draw the hemolymph. The hemolymph samples were diluted in 40 μl of TBS, and 20 μl were stained with trichrome and viewed by light microscopy (Table 1).

Table 1. Source of secretions and their treatments. C=calcofluor, T=trichrome.

Source	Number samples Pooled/ Replicate		Diluent Type	Volume (µl)	Slide Volume (µl)	Stain
Adults						
crop fluid	15	workers	TBS	20	10	T
fecal fluid	0.1 g	workers	TBS	300	60	C
hemolymph	15	workers	TBS	40	20	T
Larvae						
anal fluid	40	larvae	TBS/H$_2$0	20	20	C
oral fluid	15	larvae	TBS	120	60	T
midgut	15	larvae	TBS	500	60	T and C
meconium	40	larvae	TBS	500	60	T and C
exuviae	15	larvae	TBS	500	60	C
hemolymph	15	larvae	TBS	40	20	T
Dead ants + debris						
Midden pile	0.5 g	mixture	TBS	500	60	C

Adult fecal fluids, which consisted primarily of more solid fecal materials, were obtained by transferring about 0.1 g of randomly collected adult foraging workers from each colony into a 1.7 ml microcentrifuge tube. By collecting foragers, these adult workers would be expected to have empty buccal cavities, or buccal cavities filled with materials from outside of the nest. This way, they would not release the buccal pellets collected from larvae and other workers onto the inner walls of the microcentrifuge tube, ensuring that the secretions were primarily fecal fluids. Several holes were drilled on top of the lid to allow ventilation, and the ants were allowed to defecate in the tube overnight. Ants were removed, macerated in TBS, and, subsequently checked for spores by trichrome staining. The fecal materials were suspended in 300 µl of TBS and 60 µl aliquots were calcofluor stained and evaluated microscopically for the presence of spores (Table 1).

2.2.3 Collection of larval secretions

The possible sources of spores from larvae include both oral and anal fluids, but could also be associated with the meconium and the cuticle that are shed prior to and during pupation, respectively. Fourth instar larvae (Petralia and Vinson, 1979) were used for all larval sample collections. Anal fluid samples were pooled from 40 larvae per colony. To extract the anal fluid, the side of a sterile, bent insect pin was used to gently tap the venter of the abdomen. A 0.5 µl capillary tube was used to collect the fluid that was exuded from the larval anal pore. Each pooled sample was diluted with 20 µl of TBS (Table 1). The whole volume was stained with calcofluor, and viewed under fluorescent microscopy.

Oral fluids were collected and pooled from 15 larvae per colony. To extract the oral fluids from larvae, a small pellet of dried, powdered milk (about 1 mm) was gently placed immediately below the mandibles in the food basket of each larva to encourage salivary secretion and crop regurgitation (Petralia and Vinson, 1980). The pellets were allowed to remain with the larvae for 30 min, collected with sterile forceps, and transferred into microcentrifuge tubes. The pooled pellets were dissolved in 120 µl of TBS. Sixty µl aliquots were stained with modified trichrome stain and viewed by light microscopy (Table 1).

Meconia were collected and pooled from 40 larvae per colony. Mature larvae were allowed to initiate pupation in 10-cm (diameter) sterile plastic Petri dishes. On the second day, each Petri dish was screened for any larvae with partially extruded meconia, which is typical in the absence of adult workers (Lamon and Topoff, 1985). The meconium from each larva was carefully removed using two pairs of sharp, sterile forceps and carefully transferred into a 1.7 ml microcentrifuge tube. The pooled meconial pellets were macerated in 500 µl TBS, and 60 µl aliquots were then stained with calcofluor or modified trichrome and viewed under fluorescent and light microscopy, respectively (Table 1).

Since meconium is derived from the stored waste materials in the midgut of larvae along with the midgut tissue that is expelled at the initiation of pupation, the midgut and its contents were examined for spores. Midguts were removed by cutting off the head and pulling the midgut from the larvae using two pairs of sterile, sharp forceps. The midguts were transferred into a 1.7 ml microcentrifuge tube, with 15 midguts pooled

per colony. Pooled midguts were macerated in 500 µl of TBS, and 60 µl aliquots were stained with both calcofluor and trichrome, and viewed by fluorescent and light microscopy respectively (Table 1).

Because meconium and midgut samples may have been contaminated with hemolymph, hemolymph samples from larvae were also evaluated for the presence of *T. solenopsae* spores. Samples of larval hemolymph were collected and pooled from 15 larvae per colony using 0.5 µl microcapillary tubes. The hemolymph was diluted into 40 µl of TBS, mixed, and 20 µl aliquots were stained with trichrome.

To collect samples of pupal exuviae, early prepupae were transferred into Petri dishes as described for the collection of meconium. The isolated brood were allowed to continue development for the next 5 days in 28°C or until the prepupae had pupated. The exuviae of 15 prepupae were collected with forceps, placed into a 1.7 ml microcentrifuge tube, and macerated in 500 µl of TBS. Sixty µl aliquots were stained with calcofluor and observed with fluorescent microscopy.

2.2.4 Collection of midden piles

About 0.5 g of midden pile material, dead ants and colony debris, were collected from the foraging area of each colony and transferred into 1.7 ml microcentrifuge tubes where 500 µl of TBS was added. The samples were macerated, and 60 µl aliquots were stained with calcofluor and observed by fluorescent microscopy.

2.3 Results

Ants from 24 infected colonies and 13 uninfected control colonies were evaluated. All

Table 2. Presence of the spores of *Thelohania solenopsae* in secretions of *Solenopsis invicta*.

Source	Controls Number colonies Examined	Infected	%	Infected Number colonies Examined	Infected	%
Adults:						
Crop fluid	10	0	0	12	2	16.7
Fecal fluid	11	0	0	16	12	75
Hemolymph	6	0	0	7	0	0
Larvae:						
Anal fluid	9	0	0	10	0	0
Oral fluid	8	0	0	11	0	0
Exuvea	3	0	0	4	0	0
Meconium	7	0	0	12	11	91.7
Midgut	7	0	0	8	7	87.5
Hemolymph	5	0	0	5	0	0
Other:						
Midden pile	10	0	0	13	13	100

field-collected colonies were determined to be infected with microsporidia by trichrome and calcofluor staining. Pathogen identity was confirmed to be *T.solenopsae* by molecular analysis using PCR amplification of a portion of the small subunit RNA gene (Snowden et al., 2001).

2.3.1 Adult ants and their secretions

Twelve of 16 (75%) infected colonies were positive for the presence of spores in the fecal fluids of adults (Table 2). In these samples, no more than 5 free spores were detected per slide. Spores were not detected in any of the fecal fluids from 11 control colonies. Free spores were detected in 2 of the 12 crop fluid samples from infected colonies (17%), while no spores were detected in the crop fluids of 10 control colonies (Table 2). No spores were detected in the hemolymph samples of any adults (Table 2), suggesting that the spores in these two positive samples originated from their respective secretions.

2.3.2 Larval secretions

Possible sources of spores that could come from larvae include the anal fluid, oral fluid, meconium, and pupal exuviae. The midgut was also examined because it is the source of meconium and some of the regurgitated oral fluid. No spores were found in the anal and oral fluids, pupal exuviae or hemolymph of larvae from any of the infected or negative control colonies (Table 2). Eighty eight percent (7 if 8 colonies) of the larval midgut samples from infected colonies contained both free- and octospores, with an average of 10+ spores per slide. Megaspores were also detected in the midgut in 6 of 8 samples (75%), which averaged about 2 megaspores per slide. No spores were

found in the control samples (N=7) (Table 2). In the meconium samples, 11 out of 12 (92%) of the infected colonies had spores in the meconial pellet (Table 2). In these meconium samples, an average of 12 spores were detected per slide, including both free- and octospores. Megaspores were also detected in the meconium in 9 out of 12 samples (75%), which averaged about 2 megaspores per slide. The absence of spores from the larval hemolymph samples suggested that the spores found in the midget and meconium did not come from hemolymph contamination during sample collection.

2.3.3 Midden piles

Midden piles contained spores of both types in high numbers (>12 per slide) in all infected samples (N=13) examined. No spores were found in control midden piles (N=10) (Table 2).

2.4 Discussion

In the adults, among all of the potential sources of secreted *T. solenopsae* spores, only the fecal fluids were found to harbor free spores. It remains to be determined if these free spores were infective, which would require feeding experiments. However, Cook (unpublished data) failed to initiate infection by including purified spores in peanut butter. Among the many problems with feeding experiments of purified spores, one is that it is not representative of what is occuring in the natural environment. That is, spores are never transmitted in nature in purified form. Therefore, feeding experiments on purified spores were not performed. A better way to test for the infectivity of adult anal fluid would be to directly feed the fluid to uninfected colonies and these studies are in progress.

Transmission of microbial symbionts via coprophagy is well documented in other insects, such as termites (Tanada and Kaya, 1993). Andreadis (1987) also showed that *Nosema pyrausta* is horizontally transmitted among corn borers by contaminated fecal materials. In honey bees infected with *Nosema apis*, Schmid-Hempel (1998) noted that workers become dysenteric and pass out *N. apis* – infected fecal materials in the nest, further enhancing the transmission of disease, whereas uninfected individuals would normally defecate outside the nest. Becnel and Andreadis (1999) suggested that the spores enclosed in fecal fluids may survive longer than spores exposed more directly to the environment. The demonstration that *T. solenopsae* free spores occur in the fecal fluids released by adult workers may suggest one route of infection to other members of the colony, as suggested by Oi et al. (2001), if free spores are confirmed to be infective. The data here, showing a low abundance of free spores in the adult fecal materials, would be consistent with the data of Oi et al. (2001) indicating low rates of horizontal transmission when only infected workers are present. In the case of *N. apis*-infected honey bees, the midguts are significantly damaged (Schmid-Hempel, 1998). Whether or not this is also true in the case of *T. solenopsae*-infected fire ants remains unknown, because the integrity of the digestive system of adult *S. invicta* during of infection has not been assayed. Because spores were detected in the adult fecal materials, the possibility that the midguts are damaged due to infection cannot be eliminated. Therefore, it will be important to assay for the presence of spores in the midgut and document the extent of damage of the gastrointestinal system by this parasiteand these studies are currently underway.

In several colonies, a small number of spores were detected in crop fluid; howeverthe importance of these spores is unclear.   Seven infected colonies were examined for spores in the hemolymph because the crop fluid could have been contaminated by spores in the hemolymph, and no spores were found. This suggested that the spores in the crop fluid did not come from hemolymph contamination during sample collection. Another question is whether these spores in the crop fluid may be regurgitated. Glancey et al. (1981) showed that workers prevent particles larger than approximately 0.88 μm from being swallowed. Free spores and each of the octospores are 5 and 2 μm, respectively, in size, so both spore types are much larger than the buccal filter in the adult workers. Further, whether this inability to ingest particles larger than 0.88 μm is unidirectional is unknown. Thus, whether such workers, via tropholaxis, may be the resource of spores is unclear. Because of this particle size limitation of workers, the source of the spores in the crop fluid remains unclear. Because adult workers are unable to swallow spores, the spores in the crop fluid may come from the spores that the workers had as larvae, which do not have the buccal filter. Regardless, the low incidence of spores in the crop contents of workers and their low concentration in the crop suggest that regurgitation of crop fluid by adults may not be an important route of transmission.

Potential sources of larval transmission, besides presumed cannibalism (Sorensen and Vinson, 1982), include oral fluid, anal fluid, meconium, and the shed cuticles associated with the prepupal molt. Because our data suggest that meconia are the only exudate to contain spores, these exudates may be the candidates for horizontal

transmission. The spores in both midgut and the meconium were not likely from contaminated hemolymph because the hemolymph samples did not contain any spores. Whereas only one spore type, the free spores, was detected in the adult anal fluid, three types of spores, namely megaspores, free spores, and octospores were detected in both the larval midguts and meconia. The infectivity of each type of spores was not determined as it is beyond the scope of this project.

Oi et al. (2001) found that, in addition to the infected larvae, the workers must also be present to transmit the disease to other colony members. Based on this finding, they suggested that something must be transferred between the infected larvae and the rest of the colony. Our data suggest that the meconium from infected larvae might play a key role. Cassill and Vinson (unpublished data) have discovered that workers remove and imbibe the meconium, formerly the midgut of the developing larvae, shed from prepupating fourth instar larvae. As they imbibe the liquid content of the meconia, their gasters enlarge noticeably, suggesting that the workers were taking in materials from the meconia. These nurses then feed other members of the colony with the materials obtained from the meconia. Based on the above data, an experiment under way is to test the transmissibility of spores through artificially extracted meconium. If the meconium were indeed the sole larval exudate responsible for disseminating the disease, then the artificial inoculation of uninfected colonies with the meconium should produce infected colonies.

Since spores are too large to be consumed by most workers, several questions arise. Do spores accumulate in the buccal cavity as they imbibe the contents of the

meconia because of the inability of workers to swallow particles larger than 0.88 μm? If so, would they feed the accumulated spores to larvae or queens, both of which may be able to consume spores? These questions are under study. If these hypotheses were true that the spores do indeed accumulate in the buccal cavity, the manner of spore dissemination may be comparable to the methods of transmission of *Burenella dimorpha*. Whereas *B. dimorpha* is known to spread by accumulation and subsequent dissemination of spores in the buccal cavity by the cannibalism of moribund hosts (Jouvenaz et al., 1981), *T. solenopsae* may be horizontally transmissible by the accumulation of spores in the buccal cavity by the ingestion of spores from the meconium.

Because spores were found in the midden piles, these materials may serve as a potential source of spore dissemination into the environment. The midden piles were made of waste materials of various kinds, including dead ants (Hölldobler and Wilson, 1990). Spores may be disseminated into the environment when the host dies and decomposes. This idea has been suggested (Becnel and Andreadis, 1999). As new colonies nest in contaminated soil, they may, in turn, become infected because workers may become infected by ingesting contaminated soil as they excavate it. Although this has not been directly shown in this species, this does follow in accordance to the predictions by Becnel and Andreadis (1999, Fig. 21).

The brood may become agents of intercolonial transmission during brood raiding (Williams et al., 1999; Oi and Williams, 2002) because, according Tschinkel (1992), after a brood raiding event, the victorious colony invades and takes the brood of the losing colony to use as their slaves. Because infected colonies are usually less vigorous

at defending their nests (Mitchell, personal communication), they become losers in a brood raiding event. If this happens, the uninfected winners of this event then transfer the infected brood from the infected colony that lost the brood raiding contest. Under this scenario brood cannibalism is unlikely (Tschinkel, 1992). Thus the question becomes how the spores enter the colony and infect other colony members. Our data suggest that, as these captured slaves pupate, they may release the meconia filled with spores. The workers may unknowingly share these infected materials with other members of their own colony, thus infecting them. These may be the scenario of horizontal transmission involving brood raiding.

T. solenopsae spores of various types were found in several of the fire ant secretions. This finding may provide further mechanistic support and explanation of Oi et al. (2001), which showed that infected larvae cannot infect a colony without the aid of workers. The authors suggested that contaminated secretions from larvae might be responsible for horizontal transmission of spores. In the larvae, the secretions that may be important in the horizontal transmission of this disease could include the meconium not only because the meconia were the only larval exudates found to carry spores, but also because of the particular nature of processing this material in the fire ant colonies. The nature of the spores in the crop fluid of adult ants remains uncertain because of the uncertain source of these spores and the uncertainty of whether or not the spores may go both ways through the filter. The spores found in the anal secretions of adults might be attributed to a direct transmission from adult workers to a colony, without the brood because of the common nature of producteal feeding in insects. This finding also

provides mechanistic explanation of Oi et al. (2001) because these authors found that infected workers alone, without larvae, may also initiate infection at very low rates. Also, the presence of the spores in the anal fluid may further suggest damage of the infected midguts in the adults, which will require further study. Finally, the midden piles may be important because of the possibility of dissemination of spores into the environment due to the breakdown of infected tissues.

CHAPTER III

EFFECTS OF *Thelohania solenopsae* (MICROSPORA: THELOHANIIDAE)

PARASITISM ON THE HEMOLYMPH PROTEINS IN THE FIRE ANT,

Solenopsis invicta ALATES AND DEALATES

3.1 Introduction

Red Imported fire ants were accidentally brought to North America in the 1940's (Lofgren, 1986). Since then, due to the lack of natural enemies, their aggressive and opportunistic behavior, and their high reproductive rates, these ants have quickly spread to the Southeastern states and California (Dowell and Krass, 1992). Due to their painful stings resulting from contact, particularly nest disturbance, these ants have become serious medical and agricultural pests

As a part of an effort to develop a biological control approach for their management, the microsporidia *Thelohania solenopsae* has been identified as a potential biological control agent (Briano and Williams, 1997; Briano et al., 1995a). Both laboratory and field colonies, when infected with this pathogen, decline significantly over time (Briano et al., 1995c; Williams et al., 1999). These pathogens were first discovered from infected ants in Brazil (Allen and Buren, 1974) and later in North America (Williams et al., 1998). Because of their recent discovery, many aspects of the pathogen and the pathogen-host interaction remain enigmatic. Most of the pathogen-host interactions in this system have been descriptive in nature (Knell et al., 1977; Jouvenaz, 1983; 1986; Patterson and Briano 1990; Briano and Patterson, 1994; Moser et

al., 1996; Briano and Williams, 1997; Sokolova and Fuxa, 2001), and little study of the interactions at the molecular level has been undertaken.

Effects due to pathogens and parasites on the hemolymph of their host have been investigated in many insects. For example, novel proteins have been found in hosts parasitized by some insect parasitoids (Sroka and Vinson, 1978; Cook et al., 1984; Harwood et al., 1994). Contrary to other parasites and pathogens, physiological effects on insect hosts due to microsporidia infection have rarely been investigated (Weidner et al., 1999). Of the few reports, Hoch et al. (2002) documented changes in the carbohydrate and fatty acid levels in the hemolymph of *Lymentria dispar* infected with a microsporidia. Henn et al. (1998) documented changes in the total amino acid composition of the adult and larval dipteran, *Anopheles stephensi,* due to infection by *Nosema algerae*. Because of the manner in which the samples were prepared (whole-insect maceration), it is uncertain whether these amino acid differences were from the host or the pathogen. In the cricket, *Gryllus bimaculatus*, Sokolova et al. (2000) found the presence of the microsporidia *Nosema grylli* in the hemocytes of the host. Infected hemocytes became deformed and resemble xenomas, filled with spores.

The fat body is one of the primary sites of microsporidia infection in insects (Becnel and Andreadis, 1999), and is the major contributor of proteins found in the hemolymph (Keeley 1985, Hanost et al., 1990). Infected tissues often show gross morphological and histological changes. Fat body tissues of *Locusta migratoria* infected with *Nosema locustae* have a whitish appearance whereas fat body tissues of uninfected individuals are yellowish. Weissenberg (1976) noted that spores may eventually displace

the cytoplasm of the fat body cells of the larval cranefly, *Tipula literalis*. According to

Tanada and Kaya (1993), microsporidia spores eventually replace all of the cytoplasmic

organelles except for mitochondria and the nucleus. They also noted that infected cells

may eventually lose their function. In the case of fire ants, infected fat body tissues

became filled with spores containing cysts (Allen and Buren, 1974; Knell et al., 1977).

Thus, during the course of infection, the fat body tissues are severely damaged, which

may then adversely affect host metabolism (Hoch et al., 2002). Although the

microsporidia do not directly inhibit the hemolymph, they may, in light of these findings,

affect the humoral proteins through their effects on the fat body tissues. If such changes

occur early, they might provide early indicator of infection and provide insights into the

development of the disease.

3.2 Materials and methods

3.2.1 Ants and rearing

Ants were collected from several sites in the Brazos County, TX by transferring

the nest materials to buckets using shovels. The collected nests were subsequently

brought to the laboratory. After 48 hr of holding in the laboratory, the ants were

separated from the nesting material by the drip-flotation method (Jouvenaz et al., 1977;

Chapter II). Using methods of Greenberg et al. (1985), the ants were classified to be

either monogyne or polygyne. The ants were reared by previously described methods

(Chen and Vinson, 1999; Chapter II). Colonies were screened to be *Thelohania* –

positive or negative by modified trichrome staining (Weber et al., 1992; Chapter II) and

Calcofluor – M2R White staining (Didier et al., 1995; Chapter II). To screen the

colonies, 500 µl of TBS (Tris-buffered saline) and 60 µl of aliquots of the homogenate were used for each of the staining methods.

3.2.2 Hemolymph samples

Hemolymph samples were collected from queens (both alates and dealates) in accordance to Lewis et al., (2001). Briefly, queens were removed from their colonies using sterile forceps and immobilized by CO_2. The immobilized queens were sterilized by dipping into 95% ethanol, dried on Kim-wipe, placed onto a wax block, and fixed onto position. ventral side face up, by a staple placed between the thorax and the gaster. A sterile, sharp insect pin was used to puncture the intersegmental membrane between the second and third tergites of the gaster. The hemolymph was collected from individual queens using 0.5 µl microcapillary tubes (Alltech Associates, Inc., Deerfield, Il). The collected samples were immediately placed into 9.5 µl of cold TBS, and the queens were immediately dissected to determine if they were inseminated or uninseminated by looking at the spermatheca. The spermatheca of inseminated queens is opaque white while it is clear in uninseminated queens. In addition, the infection status of the dissected queens was determined by macerating the carcass in 500 µl ddH_2O, or double deionized water. Twenty µl of the homogenate were spotted onto a microscope slide that was scanned at 400X magnification for spores. The following schemes were used to classify the hemolymph samples. The infection status of the source colonies were labeled as U=uninfected controls, and I=infected colonies. Next, the reproductives were removed and the winged status of the queens were classified as A=alate or D=dealate. Following hemolymph collection, the insemination status of

these queens was further classified as M=mated or V=virgin and the infection status of

the specific queens were classified as n=no spores or s=spores.

The pooled samples (total of 50 μl) consisting of five females of the same

classification were centrifuged to spin down the hemocytes, 45μl of the supernatant was

recovered, from which 5μl was removed to determine the protein concentrations using

the Total Protein method (Sigma Diagnostics, St. Louis, MO), according to the

manufacturers instructions. Forty microliters of 2X sample buffer [5% SDS (w/v);

0.126M Tris-Cl; pH = 6.8; 12.5% (w/v) glycerol; 0.004% bromophenol blue, 2% (w/v)

NaCl; 10% (v/v) β–mercaptoethanol] (Lewis et al., 2001), was added to the remaining

40 μl of the samples which were then subjected to sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

3.2.3 SDS-PAGE analysis

Initially, any differences in hemolymph proteins related to infection, particularly

the appearance of a novel protein or peptide in the hemolymph due to infection were

determined. To increase chances to visualize any differences, the total hemolymph

protein samples were divided and adjusted to 2 and 16 μg of total protein, according to

the results of the Total Protein assay (Sigma). Proteins of two total concentrations were

used because the low concentration was used to visualize proteins of high concentrations

while the low concentration of total protein was used to visualize proteins of high

concentration. The samples were then adjusted to the same volume (30μl) by adding 1X

sample buffer to load into the gels.   1X sample buffer was obtained by diluting equal

volumes of 2X buffer with mili-Q water. SDS-PAGE was performed according to Laemmli (1970) with 10% bis-acrylamide resolving gels and 4% stacking gels. Gels were made according to the instructions of Sambrook et al., (1989). Standard sized gels were made using Biorad apparatus. Electrophoresis was performed at 200 volts of constant power until the tracking dye migrated off the gel. The gels were stained with silver, and the reagents of which were made according to Blum et al (1987). Electophoretic separations of the proteins were performed on three different samples from the same type of treatment conditions. Bio-Rad Prestained SDS-PAGE Standards, Broad Range was used as protein staindards. In all gels, 5 μg of the standards was used.

3.2.4 Vitellogenin (Vg) titers

Another effect of the disease may be to quantitatively affect protein titers in the hemolymph. However, since silver staining is not ideal for measuring protein titers due to its oversensitivity(Coates, personal communication), the concentrations of the protein/peptide bands in the silver–stained gels (above) were not determined. However, due to its importance as one of the major proteins secreted by adult fat body tissues (Lewis et al., 2001) and high expression of vitellogenin, the titer of Vg-I in the hemolymph was determined. Determination of Vg in the hemolymph was also important because it is also found in alates and mated and virgin dealates (Vargo and Laurel, 1994). The Vg-I concentration was determined according to the methods used by Vargo and Laurel (1994) and Lewis et al., (2001). Briefly, 0.5 μl of hemolymph samples were extracted from each of the 8 infected and 10 control queens as described above (the infection status was determined after sample collection as described above).

The hemolymph sample was immediately placed into 4.5 μl of ice-cold TBS (Tris-Buffered Saline, pH 7.4) and mixed with 5 μl of 2X running buffer. Fresh eggs laid by queens from control colonies within 4 hr of oviposition were macerated in 9.5 μl of 1X running buffer and were used to confirm the location of the Vg-1 band. The samples were electrophoresed on a 10% resolving gel with a 4% stacking gel. The gels were then stained with Coomassie Brilliant Blue (Davis et al., 1994). Video densitometry was performed in accordance with Vargo and Laurel (1994), using known concentrations of Bovine Serum Albumin (BSA) as a reference standard.

3.2.5 Fat body types and infected cells

To observe the fat body tissues, each queen was dissected on a wax block in TBS. During dissection, the guts, ovaries, poison gland and reservoir, and spermatheca were removed. A sample of the TBS in which the queens were dissected was used to determine infection state using brightfield microscopy at 400X magnification by looking for the presence of spores in the medium. The fat body tissues were then removed and transferred to a microscope slide for observation under brightfield microscopy at 400X. The fat bodies were removed by placing each gaster ventral side up and fixed in place using a staple in a wax block lined with parafilm. Two pairs of dissecting forceps were used to carefully remove the fat bodies. The dissected fat body tissues were transferred into eppendorf tubes for storage using pipetters equipped with wide pore pipet tips. In microscopy, fat bodies were transferred from eppendorf tubes to microscope slides. Seven fields per queen were observed in microscopy. In each field, the following were recorded: the number of clear trophocytes and the number of trophocytes with lipid

droplets, and the number of *T. solenopsae*-filled cells. Sample size was 10 uninfected queens and 11 infected queens.

Because visual detection of spores may not always be reliable without staining (Didier et al., 1995), attempts were made to stain infected cells with calcofluor white M2R (Didier et al., 1995) to aid the differentiation of these cells from uninfected cells surrounding them. To investigate the extent of infection in the fat body tissues, fat bodies were extracted from infected inseminated dealates and uninfected controls. Briefly, queens were fixed in place on a wax block lined with parafilm and checked for insemination as before. Only the inseminated queens were examined in this part of the experiment. 200µl of PBS (phosphate-buffered saline, Sambrook et al., (1989)) was added. The venter of the gaster was then carefully cut open and the internal organs, including the mid- and hindguts, spermatheca, poison gland and reservoir, and ovaries were removed. Immediately after the removal of internal organs, the PBS in which the queen was dissected was checked for presence of *T. solenopsae* spores as described before. Only queens that have spores in the buffer were used. The gasters, containing fat body tissues, were washed once with 2% formalin and fixed in clean 2% formalin for 4 hours. After the formalin fixation, the gasters were further fixed in methanol for 10 minutes. The doubly fixed gasters were later stained with calcofluor white M2R (Didier et al., 1995; Chapter II). Briefly, the methanol-fixed gasters were stained with Calcofluor White M2R for 3 minutes, washed with water, counterstained with 0.1% Evan's Blue., and washed with water. The fat bodies from each queen were then carefully removed from the gasters in PBS and transferred into an eppendorf tube with

200μl PBS using wide-pore pipet tips for storage. The fat bodies were visualized using

an Olympus fluorescent microscope (Model BX-51), equipped with a digital camera

(Hammatsu Co., Tokyo, Japan) linked to a custom-built PC (C-Imaging Systems,

Compix, Inc., Cranberry Township, PA). The fat bodies were imaged using the software

Simple PCI (Compix, Inc.) For the fat body samples of each queen, five images were

taken at various locations of the tissues at 200X. Both bright field and fluorescent

(excitation wavelength = 350nm; emission wavelength=470nm) images were taken at

the same location of the tissue. The bright field image was subsequently used to count

the total number of cells while the fluorescent images were used to count the total

number of infected cells.

3.3 Data analysis

In the section of humoral proteins comparison, the three replicate gels were first

compared for consistency in protein bands, number, and location. Then a comparison

was made between treatments. Protein bands that were consistently missing or new

bands that were consistently expressed in all three replicates gels compared to the

controls were noted. The molecular weight of such bands were estimated by using the

AlphaImager software (Alpha Innotech Corp., San Leandro, CA) based on plots of

semilog vs. distance based on the marker lane.

The Vg I titers were calculated based on the BSA standard using the

AlphaImager software (Alpha Innotech Corp., San Leandro, CA). Averages of the 10

uninfected and 8 infected samples were compared using the t-test. In the observations of

fat body cells, the sum of each type of cells of the seven fields was calculated. Also, the

percentage of each type of trophocytes was calculated.

3.4 Results

In this investigation, an attempt was made to determine any proteins that were

unique in either infected or uninfected queens. The initial focus was only on the global

pattern of differences in hemolymph protein profile such as number of proteins

identified and their molecular weights.

There were differences in the number of protein bands resolved in silver stained

SDS PAGE gels from the different types of females (Table 3). Among the uninfected

control colonies, the virgin alates (U, AV) had an average of 35 total bands resolved

(N=3), the highest of all samples. The uninfected virgin alates from infected colonies (I,

AVn) had an average of 33 protein bands (N=3), while infected virgin (I, AVs) had an

average of 25.33 protein bands (N=3). An ANOVA test of the number of bands in the

three replicates of the three states of alates revealed significant difference among (Table

3). It should be noted that the females from infected colonies that were labeled as

uninfected may be infected either in an earlier stage prior to spore production or had

parasitemia below the threshold of detection by direct visual observation.

The uninseminated dealates from uninfected colonies (U, DV) had an average of

33.67 protein bands (N=3), while uninfected queens from infected colonies (I, DVn) had

an average 34.67 protein bands (N=3). In contrast, the infected uninseminated dealates

(I, DVs) had an average 22.33 protein bands (N=3). An ANOVA test of the number of

Table 3. Average (N=3) number of protein bands in the samples and an ANOVA between uninfected (U) and infected (I) females from infected colonies (n) and infected females from infected colonies (s). A=Alate, D=dealate, V=virgins, M=mated, I=infected colonies.

	Colony Type						
Queen Type	Uninfected (U)	Infected (I)		ANOVA			
		n	s	df	F	p	F(crit)
AV	35	33	25.3	2	20.67647	0.0007779	6.944276
DV	33.7	34.7	22.3	2	25.85714	0.005155	6.944276
DM	32	31	27.7	2	10.69231	0.02483	6.944276

bands in the 3 replicates of the three states of these dealates revealed significant

differences (Table 3)

In the inseminated dealates, the uninfected controls (U, DM) had an average of

32 protein bands (N=3). The uninfected queens from infected colonies (I, DMn) had 31

protein bands (N=3). Finally, the infected, inseminated queens (I, DMs) had an average

27.67 protein bands (N=3). An ANOVA test of the number of bands in the 3 replicates

of the three states of these dealates revealed significant difference among them (Table 3).

However, due to the inclusion of "uninfected" samples from infected colonies in

the analyses could have been contaminated with unknown number of infected

individuals with an infection below the threshold of detection , we removed the

"uninfected" sample data collected from infected colonies and repeated the analysis.

Infected vs. uninfected inseminated dealates, infected vs. uninfected uninseminated

dealates, and infected and uninfected alates were also compared. The differences in the

number of protein bands between infected and control samples in this analysis were

highly significant in all cases (Figure 1).

3.4.1 Specific comparisons

The proteins on the SDS PAGE gels were separated from the three different

states of females from uninfected and control colonies. The three states included alates,

uninseminated dealates, and inseminated dealates. Comparisons were made between

infected and uninfected samples on the same gels and between the three replicate

samples of the same conditions on a gel three times with different samples. The three

replicate gels of the same comparison were then searched for the same bands.

Comparison of Avg. No. of Bands Between Infected and Uninfected Queens

ANOVA

df	F	P-value	F crit
5	21.16	5.04E-05	3.33

Figure 1. Comparison of all controls and infected samples. Silver stained SDS PAGE gels. UAVn: Uinfected virgin alates; IAVi: infected virgin alates; UDVn: uninfected, vigin dealates, IDVi: infected virgin dealates; UDMn: uninfected, mated dealates; IDMi: infected, mated dealates

In the samples for alates, we compared between the uninfected and the infected samples. Although there was some variation among the three gels in this comparison, we nevertheless were able to identify 6 bands that were present among all three control replicate gels, but absent in the infected samples. The estimated molecular weights of these 6 protein bands were 132, 115, 114, 110, 45, and 42 KD (Figure 2). No protein band was found to be unique in infected samples.

In the samples for uninseminated dealates, the comparison was between the banding patterns controls and infected. Four protein bands were identified in the controls but absent in the infected sample (Figure 3). These were 102, 100, 74, and 44 KD. No protein band was found that to be present only in the infected samples

In the samples for inseminated dealates, comparison was made on the banding patterns of U, DM controls and infected. Four protein bands were shared by all three gels and identified only in the controls. These bands had the molecular weights, 111, 72, 43, and 32 KD. In the comparison that looked for protein bands in the infected samples but not in the controls, one unique band was found at 35 KD. This band was shared by all three gels. (Figure 4)

3.4.2 Vitellogenin I (Vg I) concentration comparison

With a sample size of 15 controls and 12 infected dealates, a highly significant difference was found (Figure 5) in the Vg I titer that occurred at 182 KD (Lewis et al., 2001). Whereas the uninfected dealated queens had approximately 46µg of total proteins per µl of hemolymph, the infected individuals had approximately 15 µg per µl of hemolymph.

Figure 2. Comparison of the alate samples from controls and infected samples. Silver stained SDS PAGE gel. Lane 1: Marker; lane 2: alates from uninfected colonies, 2 μg, lane 3: alates from uninfected colonies, 16 μg; lane 4, infected alates, 2 μg, Lane 5: infected alates, 16 μg. Arrows in the control lane point to the protein bands that were present in the controls but absent in the infected samples. These protein bands were the consistent protein bands from all three gels.

54

Figure 3. Comparison of the uninseminated control dealate samples and the uninseminated, infected dealate samples. Silver stained SDS PAGE gel. Lane 1: marker; lane 2: uninfected, uninseminated dealates, 2 μg; lane 3: uninfected, uninseminated dealates, 16 μg; lane 4: infected, uninseminated dealates, 2 μg; lane 5: infected, uninseminated dealates, 16 μg. Arrows in the control lane point to the protein bands that were present in the controls but absent in the infected samples. These protein bands were the consistent in all three gels.

Figure 4. Comparison of inseminated control samples and inseminated infected samples. Silver stained SDS PAGE gel. Lane 1: marker; lane 2: uninfected, inseminated dealate, 2 μg; lane 3: uninfected, inseminated dealate, 16μg; lane 4: infected, inseminated dealates, 2 μg; lane 5: infected, inseminated dealates, 16 μg. Arrows in the control lane point to the protein bands that were present in the controls but absent in the infected samples. The sole arrow in the infected lane (5) indicated the unique band that was present in the infected samples. These protein bands were the consistent from all three gel.

Figure 5. Comparison of the titres of hemolymph vitellogenin between healthy and infected queens in μg/μl hemolymph.

a

b

Figure 6. Infected fat body cells, 200X. a: Infected fat body cells, 200X, bright field. The arrows point to infected cells. White bar = 100μm. b: Infected cell, calcoflour stained, fluorescent view, 200X. The white arrows point to infected cells. Uninfected cells were not stainied. White bar = 100 μm.

3.4.3 Fat body observations

In brightfield microscopic observations without staining, no parasite-infected cells were found in any of the control queens (N=10). Infected cells were detected in 9 of 11 infected queens (82%). Figure 6a shows a sample of infected tissue in bright field microscopy. In these infected queens, infected cells were visualized in 10% of the total fat body cells. In the case of infected fat body cells stained with calcofluor, the percent of infected cells was 15%. Figure 6b shows the same image, calcofluor stained and viewed under fluorescence..

In addition to infected cells in infected queens, the other types of cells observed in the fat body tissues included trophocytes without lipid droplets and trophocytes with lipid droplets. In the uninfected queens, trophocytes with lipid droplets consisted of 45.6% of total cells, while clear trophocytes without lipid droplets consist of 53.4% of total cells. In the case of infected queens, trophocytes with lipid droplets consist of 32.6% of the total cells while clear trophocytes without lipid droplets consist of 57.5% of the total cells examined (Figure 7). The remaining 10% of the cells were infected, as described above, according to bright field microscopy.

When the percentages of cells of each type were compared, there were no significant differences between the percentage of clear trophocytes and trophocytes with lipid droplets (Figure 6, error bars) in uninfected controls. However, there was an insignificant decline in the number of trophocytes with lipid droplets in the case of infected samples.

Percentage of Each Trophocyte Type

Figure 7. The percentages of types of trophocytes in fire ants. There were two types of trophocytes examined in this experiment. These included the trophocytes with lipid droplets (LD Cells), and no droplets (Clear Cells). Also, infected cells (Inf. Cells) were detected in infected fire ant queens. Cells were counted using bright field microscopy.

3.5 Discussion

Studies concerning an insect host's humoral response to pathogen infection or parasitoids have demonstrated that there are changes to the the host's hemolymph proteins, including the up regulation of some proteins, elicited by the presence of the foreign invaders (Sroka and Vinson, 1978; Cook et al., 1984; Tanada and Kaya, 1993; Rolle and Lawrence, 1994; Boucias and Pendland, 1999; Boulanger et al., 2001; Ouedraogo et al., 2002). Although there are few studies concerning the effects of pathogens or parasitoids on ants, Makintosh et al. (1998) reported activation of O-glycosylated antibacterial peptides in the ant *Myrmecia gulosa*. In contrast, fewer studies concerning the effects of pathogens on the hemolymph proteins of their hosts have demonstrated the down regulation of select peptides and proteins (Tanada and Kaya, 1993; Jarosz and Glinski, 1990). Specifically, Jarosz and Glinski (1990) documented down regulation of a humoral protein by a compound isolated from the scales of American foulbrood, *Bacillus larvae*.

Most of the studies concerning the effects of pathogenic infection on the hemolymph proteins of their insect hosts have focused on the humoral immune response. However, because *T. solenopsae* are intracellular pathogens, they may be less likely to induce the humoral immune response, unless released into the body cavity due to the destruction of infected tissues (Becnel and Andreadis, 1999). In the few studies dealing with changes in hemolymph physiology due to microsporidia infection, Weidner et al., (1999) documented an increase in lactate concentration in the hemolymph of the blue crab *Callinectes sapidus* due to the infection by the microsporidian, *Ameson michaelis*.

Hoch et al., (2002) noted a decrease in the titer of trehalose, fatty acids, and carbohydrates in the *Lymentria dispar* larvae that were infected by the microsporidian *Vairimorpha sp.* Overton (2003) examined the effects of *T. solenopsae* on the red imported fire ants (RIFA) using whole body extracts and found that the total proteins of infected female alates were not significantly different than uninfected controls, although there were significant differences in total lipids. However, both studies suggest that there is a depletion of the hosts' nutritional resources due to microsporidian infection.

The effects of *T. solenopsae* infection on host humoral proteins were examined and marked changes in the humoral protein profiles of infected fire ants were found. In all cases, infected samples had fewer protein bands than the controls using silver stained SDS PAGE gels. These results suggest that the infection of the fat body tissues by *T. solenopsae* has an impact on the host hemolymph protein profile. Further, many of these changes were specific to the physiological state of the queen since there were significant differences in proteins in the comparisons among infected and control alate, uninseminated dealate, and inseminated dealate females (Figures 2-4).

For the reasons cited above, females from infected colonies that did not harbor spores were not included in further studies to determine if specific proteins are up-or down- regulated due to infection. In all three types of females, there were more down regulated protein bands due to infection than up-regulated protein bands. In fact, only one protein band, at 35 KD, was found to be up regulated, and this protein was only found in the infected dealate mated females, when compared to controls (Figure 4, lane 5).

Comparison of the molecular weights of bands that were down-regulated in all three types of the infected queens (alates and both types of dealates) revealed a shared down-regulated protein(s) at approximately 42-44 KD. These protein bands were likely the same protein. Both of the infected dealate samples (inseminated and uninseminated) also revealed down regulated protein bands, one at 74 (I, VDs) and another at 72 (I, MDs) KD that are also likely to be the same protein. The down regulation of a 110 KD protein band occurred in both the infected alates and the infected, inseminated dealates. In addition to identifying down-regulated proteins, the different states of queen also had a loss of a unique protein bands that were correlated to infection. For example, the protein bands with the molecular weights of 132, 115, and 114 KD were only down regulated in infected, alate queens. In the case of infected, uninseminated dealates, two protein bands at 102 and 100 KD appeared to be uniquely down-regulated. Finally, the infected, inseminated dealates had one protein band at 32 KD that appeared to be uniquely down regulated. These data suggest that *T. solenopsae* affects the protein profiles of the different types of queens in a variable manner, although some broader effects occurred.

In the case of infected fire ant queens, a majority (9 of 11 queens dissected) had observable infected trophocytes. No infected cells were found in any of the control queens. The infection rate among the observed cells was at approximately 10 percent of the observed cells in bright field microscopy. Even when stained with calcofluor white M2R, the percentage was still low at 15%. This 15% infection rate is likely to be an underestimation because trophocytes infected with vegetative stages were not identifiable with the methods used here.

It is well known that intracellular pathogens may damage host cells. The infection by microsporidia is no exception. Although the extent of infection and damage to fat body cells of the fire ant has not been clearly defined (Allen and Buren, 1974; Knell et al., 1977), damage to host cells of other insects infected with microsporidia has been delineated. Weissenberg (1976) noted a "pronounced" hypertrophy of the fat body cells of *Tipula literalis* larvae infected with *Thelohania tipulae.* He noted that there was dissolution of cytoplasmic contents. The nuclei of infected cells also exhibited pronounced hypertrophy, enlarging at least four times their original size and fat vacuoles also disappeared from infected cells. Tanada and Kaya (1993) noted that microsporidia eventually replaced all of the cytoplasmic organelles except the nucleus and the mitochondria. Jurand et al., (1967), working with a developing microsporidia in the salivary gland cells of the fly *Sciara ocellaris*, described the replacement of the cytoplasmic organelles by the microsporidia and an increase in the number of mitochondria congregated around the spores. In the chironomid *Acricotopus lucidus* infected with *Nosema algerae*, Staiber (1994) noted that the activities of the polytene chromosomes of the salivary gland cells from the dipteran *Acricotopus lucidus* were affected by the infection, which altered the specific cell programs. Since *T. solenopsae* infects the fat body of their fire ant hosts, there will likely be some effects on the fecundity of the IFA (Knell et al., 1977).

3.5.1 Effects on vitellogenin (Vg)

Williams et al. (1999) reported a significant reduction in the fecundity of fire ant queens infected with *T. solenopsae*. The results of this project suggested that lowered

fecundity may bej due to the reduced hemolymph titer of Vg which is probably due to the down regulation of Vg production by the fat body. Hurd (2001) showed that the fecundity of *Tenebrio molitor* was reduced following tapeworm infection and that this reduction was correlated with a reduction of Vg in the hemolymph. Hurd and Webb (1997) argued that the tapeworm caused a reduction of host fecundity because the tapeworm utilized the nutrients that the host needed for egg production. Later, Hurd (2001) suggested that the tapeworm may suppress the production of Vg through the initiation of apoptosis of the ovary which reduced the production of ecdysone thus reducing Vg synthesis in the fat body. Whereas these previous studies documented the reduction of Vg by an extracellular pathogen's impact on the ovary suppressing Vg production in the fat body, this study dealt with a pathogen that directly parasitizes the fat body cells, the producer of Vg. Although the exact mechanism of this reduction remains unclear, data here may suggest parasitic reduction of humoral Vg by directly parasitizing and disrupting the source tissue. This proposed mechanism has some important implications. Because only as much as 16% (by Calcofluor M2R white staining) of the total cells were infected with this pathogen, the finding that infection led to such a significant decline in the Vg in the hemolymph may suggest that the effect of the pathogen is more than only the cells that carry them and that the parasites influence adjacent uninfected fat body cells. Whether only some fat body cells are invaded or the Vg production is particularly suppressed by invading the fat body, *T. solenopsae* can utilize the resources of the host while allowing for some egg production and continued vertical transmission of the disease.

CHAPTER IV

Thelohania solenopsae AND APOPTOSIS OF INFECTED CELLS IN INSEMINATED

QUEENS OF THE RED IMPORTED FIRE ANT, *Solenopsis invicta*

4.1 Introduction

Microsporidia are intracellular eukaryotic pathogens commonly found in many

species of insects as well as animals of all phyla (Boucias and Pendland, 1999).

Currently there are more than 1000 species of these parasites found in 144 genera.

Several of these are found to infect social insects such as bees and ants. In the case of

the red imported fire ants, *Solenopsis invicta*, the most prevalent microsporidia is

Thelohania solenopsae.

Because of their nature as intracellular parasites, the effects of these pathogens

on their host cells have been described in various species of host insects. For example,

Weissenberg (1976), documented that, in the case of *Tipula literalis* infected with

Thelohania tipulae, the infected fat body cells become hypertrophied, filled with spores.

Also, the infected cells, with the exception of the nucleus and the mitochondria, are

known to have their cytoplasmic organelles replaced by the spores. In the case of

Nosema apis infection of honey bees, the infected midguts**,** Liu (1984) found that

infected midgut cells became filled with spores and possessed large vacuoles. Also,

these cells had aggregated ribosomes and showed extensive lysis. Becnel and Andreadis

(1999) noted several different types of change in the morphology in microsporidia

infected cells. Aside from cellular hypertrophy, which they described as having stages

of the pathogen completely fill the host cell, they also noted several types of

relationships between microsporidia and the host cells. In the case of grasshoppers

infected of *Nosema locustae*, Becnel and Andreadis (1999) also noted gross changes of

the infected fat body tissues observable via the naked eye. In the infection of the potato

flour beetle, *Leptinotarsa decemlineata* by the microsporidia *Endoreticulatus fidelis*, the

infected midgut epithelial cells eventually rupture and the spores are then released into

the environment along with fecal materals.

In the insect system, apoptosis during development has been studied extensively

on *D. melanogaster* (e. g. Coffman, 2003; Mirkovic et al., 2002; and Richardson and

Kumar, 2002). In the case of pathogen infection, most current research on apoptosis has

been done on several species of insects on various types of pathogens, including honey

bees (*Paenibacillus larvae*) (Gregorc and Bowen, 2000), various species of

lepidopterans (baculoviruses) (Clem, 2001), and *Photorhabdus luminescens* infection of

Manduca sexta (Silva et al., 2002) In the honey bees infected with *P. larvae*, Gregorc

and Bowen (2000) characterized the apoptosis of the larval midgut after treatment with

the bacteria. In the case of *P. luminescens* infection of *M. sexta*, Silva et al. (2002)

showed that the bacteria, which are the bacteria found within the invading nematode,

actually are able to evade the immune detection of the host lepidoptera by causing

apoptosis of host's hemocytes. The effects of baculoviruses on apoptosis of the host cell

were discovered on 1991 (Clem, 2001). These viruses are known to either cause

apoptosis or, using a different pathway, inhibit it.

Other than viruses and bacteria, the effects of several intracellular pathogens on host cells have been investigated on several intracellular protozoans. For example, Nash et al. (1998) documented that *Toxoplasma gondii*-infected cells become *resistant* to apoptosis-inducing agents such as cycloheximide, UV-irradiation, pyrimethamine, and beauvericin. Whether or not microsporidia would cause the host cells to go into apoptosis was investigated by Scanlon et al., (1999). Scanlon et al., (1999) found that the microsporidia *Nosema algerae* causes the host cell, human lung fibroblasts, to have reduced susceptibility to apoptosis. More specifically, they noted that the percentage of DNA fragmentation was significantly reduced in *N. algerae*-infected cells when treated with both cycloheximide and tumor necrosis factor-α (TNF), as compared to the uninfected cells when treated with the same combination of apoptosis inducers. In this finding, these authors argued that the intracellular pathogen must prevent the premature exposure of the vegetative stages to the extracellular environment because this would be catastrophic (Scanlon et al., 1999). They also observed the ratio of *Bcl*-2 to bax. Whereas *Bcl*-2 inhibits apoptosis, bax is known to promote cell death (Cory and Adams, 2002). Scanlon et al., (1999) found that the ratio between *Bcl*-2, an inhibitor protein of apoptsosis and *bax*, an inducer protein of apoptosis, was higher in the infected cells when treated with the inducers as compared with the controls. Although microsporidia are commonly found in insects (Becnel and Andreadis, 1999), aside from electron microscopic studies of the morphology infected cells, only a handful of studies deal with the cellular response to this pathogen (e. g. Staiber, 1994). It remains unclear whether or not insect cells would undergo apoptosis when infected with this pathogen. Because

microsporidia-infected fat body cells also undergo gross hypertrophy and become

grossly enlarged cysts (Weissenberg, 1976; Becnel and Andreadis, 1999, Chapter III),

this chapter will investigate whether this change in morphology would be indicative of

apoptosis.

4.2 Materials and methods

4.2.1 Ants and rearing

Ant colonies were collected from varies sites in Brazos County, TX according to

methods used by Chen et al. (2004) and in Chapter II. The buckets containing the

colonies were brought back to the laboratory where they were held for 48 hr and were

separated from the soil by using drip floatation method as described by Jouvenaz et al.

(1977) and above in Chapter II. Ants were maintained in the laboratory by using the

protocols of Chen and Vinson (1999) and summarized in Chapter II. Colonies were

confirmed to be polygyne by using the methods used by Greenberg et al. (1985). The

infection status of each colony was determined using trichrome M2R staining (Weber et

al., 1992) and the protocols used previously (Chen et al., 2004; Chapter II, this

dissertation). The stained slides were viewed in bright field microscopy to detect spores.

4.2.2 Fat body tissue extraction and fixation, and annexin V assay

Only inseminated dealates were used in all experiments. All dissections were

performed inside a sterile laminar flow hood. Forceps and surgical scissors were all

sterilized by dipping them into 95% ethanol (EtOH) before each use. Ants were fixed

onto a wax block as described by Lewis et al. (2000) with the following modification.

Instead of directly fixing the ants onto the wax block using a staple, a piece of sterile

parafilm was placed on top of the wax block and the ants were placed in position on the parafilm. Each dealate was checked for insemination status by looking for characteristic spermatheca, which is opaque white in inseminated individuals. Uninseminated individuals were discarded. The ants were than dissected in 200 µl of insect saline (Keeley, unpublished; Appendix 1). The parafilm was replaced anew after each dissection to prevent cross-contamination.

To prepare the fat body tissue, a ventromedial cut was made on the gaster of the ant using the sharp surgical scissors. Also, the intersegmental membranes on the dorsal gaster were cut to allow the gaster to be spread open. The major internal organs, including the spermatheca, mid and hindguts, ovaries, and poison gland were removed, leaving only the fat body tissues attached to the inner wall of the cuticle. Infectious status was checked by inspecting 30 µl of the insect saline for spores.

The cuticle (gaster) was removed from the rest of the insect and washed in 200 µl incubation medium (Appendix) and stained with Annexin V and propidium iodide using ApoAlert Annexin V-FITC Apoptosis Kit (Clonetech) following the manufacturer's instructions. This kit was used because Annexin V binds to phosphatidylserine and its externalization indicates early apoptosis (Martin et al., 1995).   Briefly, the cuticles were twice washed with incubation medium (Appendix). The cutiticles were than washed once with 200 µl of binding buffer and were immersed into a fresh 200 µl binding buffer with 5 µl of Annexin V and 10 µl of propidium iodide for 10 minutes. After staining, the fat body tissues, still attached onto the cuticle, were washed four times in incubation medium (Appendix) and fixed in 2% formalin in H_2O for 4 hours. The cuticle was then

washed once with PBS and the fat body tissues were carefully removed from the cuticle in PBS and transferred into eppendorf tube containing 150 µl PBS by using a pipetter equipped with a wide-bore tip.

To image the tissues, a pipetter equipped with replaceable wide-bore tips were used to transfer the samples from eppendorf tubes to glass slides. An Olympus BX 51 Reflected Fluorescence microscope system was used. The images were digitized using a Hamamatsu Digital Camera C4741-95 connected to a computer through a Hamamatsu Camera Controller C4742-95. The computer, digital camera, and controller were from C-Imaging systems, Compix, Inc., (Cranberry Township, PA). Simple PCI software was used to analyze the images. Images were viewed at 200X magnification. In each sample, both bright field and fluorescent images were taken. In the case of fluorescence, filters for both FITC (excitation wavelength: 490 nm; emission wavelength: 525 nm) and TRITC (excitation wavelength: 557 nm; emission wavelength: 576 nm) were used to generate a composite image.

The fates of the cells (apoptosis, necrosis, dead, viable) were differentiated according to the manner of staining. Because neither propidium iodide nor annexin V can label viable cells, these cells could not be stained (Figure 15). Because apoptotic cells were stained by annexin V only, these cells appeared greenish in the fluorescent image (Figure 15). Because dead cells were stained by both labels, these cells appeared to be green cells with read nucleus (Figure 15). Finally, because necrotic cells were only stained with propidium iodide, these cells appeared to be reddish in fluorescent microscopy (Figure 16).

4.2.3 Apoptosis induction

Two methods of apoptosis induction were used. In each inducer, two time frames were performed, brief exposure and incubation. In the case of brief exposure, the dissected gaster cuticles were briefly dipped into the inducer for the inducer treatment and in the incubation medium (Appendix) for the mock treatments. First was using cycloheximide. In this investigation, seven queens were used in each treatment in this experiment. The queens were dissected and checked for infection as described. Cycloheximide was used at a final concentration 250 μg/ml. A stock solution was made by dissolving 0.25 g of cycloheximide into 5mL of absolute ethanol. In each experiment for the treatment with cycloheximide, 1 μl of the stock solution was added to 199 μl of fire ant medium (Appendix 1) (Keeley, unpublished). For cycloheximide free controls, tissues were either dipped or incubated in fire ant medium for four hours. Seven queens were used per treatment. This experiment had two parts and each part included the following treatments: uninfected cycloheximide negative, uninfected cycloheximide positive, infected cycloheximide negative, and infected cycloheximide positive. Five fields of each tissue sample were obtained at 200X magnification as images by both fluorescent and bright field microscopy. Each field had approximately 40 cells. The total number of cells counted per tissue was approximately 200. In the subsequent analysis, the following were counted: the total number of cells under going apoptosis and the total number of cells that remained viable. In the case of infected samples, in addition to counting the total number of cells undergoing apoptosis or remaining viable, the infected cells undergoing apoptosis and the number of infected cells that remained

viable were also counted. The infected cells were differentiated from non-infected cells using bright field microscopy and their unique appearance (Figure 14a). The activities of the infected cells, as determined by bright field microscopy were determined using fluorescent microscopy picture of the same image. The results were calculated as percentages.

The second test was using staurosporine (Sigma). Six queens were used in each treatment. The procedures were exactly the same as above, except staurosporine at a final concentration of $1\mu M$ was in the treatment. Stock solution at $1mM$ was prepared by using dimethylsulphoxide (DMSO) (Sigma) as solvent. During each trial 3 μl of the stock solution of staurosporine (STS) were diluted in 27 μl of DMSO to make a 1:10 dilution. This ten-fold dilution was used in each trial. In each experiment, for both the brief exposure and 1-hour incubation experiment, 2 μl of the staurosporine dilution was mixed with 198 μl of the fire ant incubation medium (Appendix). In the negative controls, 2 μl of DMSO (no STS) was mixed with 198 μl of fire ant incubation medium (Appendix). The fire ants were dissected and verified for infection as before.

4.2.4 Statistical analysis

In all statical analysis, SPSS (SPSS, Inc., Chicago, Ill.) software was used. To analyze the data, univariate general linear model was used. Also, before the general linear model was used, the data were verified to be in normal distribution using the Shapiro-Wilk Test of Normality. The assumption of equivalence of variances was tested using the Levene's Test of equality of variances. The data sets were square root transformed prior to analysis.

4.3 Results

4.3.1 Fate of cells without any treatment

In this part of the experiment, the fat body cells were extracted, stained with annexin V and fixed in formalin for 4 hr, as described. No apoptotic inducers were added in this experiment. The sample size was 7 for *Thelohania* – negative controls and 10 for *Thelohania* – positive queens. In this experiment, the controls had, in average 27.39% of apoptotic cells, 0.11% necrotic cells, 0.58% dead cells, and 71.92% viable cells. In the case of infected samples, there were 29.07% apoptotic cells, 0.41% necrotic cells, 0.16% dead cells, and 70.37% viable cells (Figure 8). In the case of infected cells, 17% of the infected cells were found to be apoptotic while 83% of the infected cells were viable. None of the cells examined was found to be either necrotic or dead. A one-way ANOVA on the percentage of cells undergoing apoptosis comparing the uninfected controls, infected tissues, and infected cells showed that the differences were not significant ($P>0.05$, $F=0.78$, $F_{crit}=3.55$). This was also true in the case of viable cells ($P>0.05$, $F=0.63$, $F_{crit}=3.55$).

Because this assay was the first experiment to stain fire ant fat body cells with annexin V, in order to differentiate between cells that were actually stained with annexin V and background fluorescence, fat body tissues from several fire ant queens were prepared and fixed as described, but without the staining . The same frames were recounted, with correction for background fluorescence. Figure 9 showed cells with background fluorescence, without staining. Also, one of the reasons to explain the low percentage of apoptotic cells in the infected cells was that there were two samples in

Figure 8. Comparison in the fates between uninfected and infected tissues in percent of total cells undergoing each fate without adjustment to background fluorescence.

which *no* apoptotic cells were observed among all of the infected cells examined. These two samples were treated as outliers and were dropped from the analysis and two more samples, which had average apoptotic cells of 10% and 20%, were used to replace these two dropped samples. The corrected data were shown in Figure 10. The adjusted results showed that the difference in percent apoptosis between infected and uninfected tissues was significant (P=0.006, t=-3.54). Because uninfected tissues did not contain infected cells, they were also used to compare with the infected cells in the infected tissues. In this comparison, the difference in percent apoptosis was insignificant (P=0.08, t=1.94). Nevertheless, the averages of apoptotic cells in the infected cells were higher than in the uninfected cells (Figure 10). The adjusted results also showed that there were overcounts (compare Figures 8 and 10) for apoptotic cells and undercounts for viable cells. The same standards were used in the subsequent assays. Figure 11 showed infected cells in the bright field view. Figure 12 showed infected cells in the fluorescent view. Figure 13 showed uninfected tissues in the fluorescent view. Figure 14 showed a necrotic cell.

4.3.2 Cycloheximide treatment

Brief exposure to cycloheximide yielded the following results. In the case of brief exposure to cycloheximide of non-infected samples (Figure 15) 13% of the cells were undergoing apoptosis. When not treated with cycloheximide, infected samples had an average of 17% of cells undergoing apoptosis. Also, in the case of brief exposure to cycloheximide, infected samples (which include both infected and uninfected cells), when treated with this agent, had an average of 19.76% cells becoming apoptotic.

a

b

Figure 9. Unstained fat body cells, 200X. a. Uninfected fat body cells, 200X. b. Unstained infected cells (arrows). Due to concerns of background fluorescence, fat body tissues from a fire ant queen were removed and fixed without annexin V and propidium iodide staining.

Figure 10. Comparison in the fates between uninfected and infected tissues in percent of total cells undergoing each fate, with adjustment to background fluorescence.

Figure 11. Infected cells, bright field, 200X. The arrows point at infected cells. White bar = 100μm.

Figure 12. Infected cells, fluorescent view, 200X. Arrows "a" point to viable infected cells while arrow "b" point to apoptotic infected cells. White bar = 100 μm.

Figure 13. Fluorescent view of uninfected tissue, 200X. Arrows "a" point to dead cells, arrows "b" point to apoptotic cells, while arrow "c" points to a viable cell. White bar = 100µm.

Figure 14. Necrotic cell, fluorescent view, 400X. Because all samples were treated with annexin V to label apoptotic cells and propidium iodide to label necrotic cells, necrotic cells were labeled red in fluorescent microscopy and they were not labeled by annexin V.

Effect of Brief Exposure to Cycloheximide

Figure 15. Comparison of percent apoptosis and viability among uninfected samples, infected samples, and infected cells in cycloheximide (brief exposure treatment). Please note that uninfected samples contain only uninfected cells while infected samples contain both infected and uninfected cells. Infected cells contain only cells that were infected with *T. solenopsae*.

Infected samples, when not treated with cycloheximide, had 16.84% of the cells undergoing apoptosis. A majority of the cells remained viable in this part of the experiment. In the case of uninfected samples, when briefly exposed to cycloheximide, 86.76% of the cells remained viable. When not exposed to this agent, 81.53% of the cells remained viable. In the case of infected samples, when briefly exposed to cycloheximide, 79.90% of the cells remained viable. When not exposed to this agent, 82.47% of the cells remained viable. In the case of *T. solenopsae*-infected cells, brief exposure to cycloheximide had the following results, as shown on Figure 15. In the cycloheximide-negative controls, 33.43% of the cells became apoptotic while 65.18% of the cells remained viable. In the cycloheximide-positive treatments, 32.06% of the cells became apoptotic while 64.98% of the cells were viable.

Exposure to cycloheximide for four hours had the following results (Figure 16). In the case of uninfected samples without the cycloheximide treatment, 10.91% of the cells were found to be apoptotic while 88.96% of the cells were found to be viable. In the case of uninfected samples with cycloheximide treatment, 10.65% of the cells became apoptotic while 89.08% of the cells remained viable. In the case of infected cells without cycloheximide treatment, 22.33% of the cells became apoptotic while 77.36% of the cells remained viable. In the case of infected samples with cycloheximide treatment, 27.31% of the cells were found to be apoptotic while 73.04% of the cells remained apoptotic. In infected cells without cycloheximide treatment, 31.09% of the cells became apoptotic, while 68.91% of the cells remained viable. In infected cells with cycloheximide treatment, 71.83% of the cells became apoptotic, while 28.27% of the

4hr Incubation Experiment, Cycloheximide

Figure 16. Comparison of percent apoptosis and viablility among uninfected samples, infected samples, and infected cells in cycloheximide (4-hour exposure treatment). Please note that uninfected samples contain only uninfected cells while infected samples contain both infected and uninfected cells. Infected cells contain only cells that were infected with *T. solenopsae*.

cells remained viable.

Two separate statistical comparisons were made using the general linear model ANOVA. Results from Levene's test of equality of variances satisfied the requirement for the equality of variances (P=0.644, F=0.734). Because the raw percentages did not fit into a normal curve, the percentages of apoptosis were square root transformed. The square root transformed data did fit the normality curve (Table 4). The first test compared between the uninfected samples and infected samples (Table 5). In this test, exposure time, infection, and cycloheximide treatment were used as fixed factors while. The dependent variable was percent apoptosis (square root transformed). The results of this test indicated that none of the factors were significant contributors to the variances in the percent apoptosis (time: P=0.855, F=0.034; inducer: P=0.426, F=0.646) (Table 5). Neither the covariate was a significant contributor to the variances in apoptosis (P=0.588, F=0.034) (Table 6). However, the infection status (infected v. not infected) was highly significant (P=0.006, F=8.373) (Table 5). The second comparison was made between infected and uninfected cells (Table 6). As in the first test, the percentages of apoptosis were square root transformed to fit a normal curve, as confirmed by Shapiro-Wilk's test (Table 7). In this case, the uninfected controls, which did not contain any infected cells, were compared with the infected cells found in the infected samples. Levene's test on equivalence of variances confirmed the validity of the general linear model ANOVA (P=0.891; F=0.410). All of the infected cells were infected. In this comparison, the percentage of apoptosis was used as a dependent

Table 4. Tests of normality in the comparison between infected and uninfected samples cyclohexximide treatment. Because there were less than 50 samples in each class of comparison, the Shapiro-Wilk test was used.

Tests of Normality

	INDUCER	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for SQRTAP	no	.126	29	.200(*)	.956	29	.264
	yes	.111	28	.200(*)	.960	28	.356

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Tests of Normality

	EXPOTIME	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for SQRTAP	low	.124	30	.200(*)	.960	30	.302
	high	.104	27	.200(*)	.963	27	.429

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Tests of Normality

	INFECT	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for SQRTAP	no	.128	30	.200(*)	.956	30	.247
	yes	.145	27	.153	.952	27	.243

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Table 5. General linear model ANOVA of cycloheximide test at the tissue level.
INDUCER: Cycloheximide; EXPOTIME: time of exposure; INFECT: infectious status
of the tissues.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
EXPOTIME	0	brief	30
	1	4 hr	27
INDUCER	0	no	29
	1	yes	28
INFECT	0	uninfected	30
	1	infected	27

Levene's Test of Equality of Error Variances^a

Dependent Variable: SQRTAP

F	df1	df2	Sig.
.734	7	49	.644

Tests the null hypothesis that the error variance of
the dependent variable is equal across groups.

a. Design:
Intercept+INFLEVEL+EXPOTIME+INDUCER
+INFECT+EXPOTIME *
INDUCER+EXPOTIME * INFECT+INDUCER
* INFECT+EXPOTIME * INDUCER * INFECT

Tests of Between-Subjects Effects

Dependent Variable: SQRTAP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	32.069^a	8	4.009	2.544	.021
Intercept	425.273	1	425.273	269.858	.000
INFLEVEL	.470	1	.470	.298	.588
EXPOTIME	.053	1	.053	.034	.855
INDUCER	1.018	1	1.018	.646	.426
INFECT	13.195	1	13.195	8.373	.006
EXPOTIME * INDUCER	.565	1	.565	.358	.552
EXPOTIME * INFECT	7.649	1	7.649	4.854	.032
INDUCER * INFECT	2.237	1	2.237	1.420	.239
EXPOTIME * INDUCER * INFECT	.023	1	.023	.015	.904
Error	75.644	48	1.576		
Total	998.393	57			
Corrected Total	107.713	56			

a. R Squared = .298 (Adjusted R Squared = .181)

Table 6: General linear model ANOVA of cycloheximide test at the cellular level. Infect: infection status; EXPTIME: experimental time: CHX: cycloheximide.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
INFECT	0	uninfected	30
	1	infected	25
EXPTIME	0	brief	29
	1	4 hr	26
CHX	0	no	27
	1	yes	28

Levene's Test of Equality of Error Variances^a

Dependent Variable: SQRTTR

F	df1	df2	Sig.
.410	7	47	.891

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept+INFECT+EXPTIME+CHX+INFECT
   * EXPTIME+INFECT * CHX+EXPTIME * CHX+INFECT
   * EXPTIME * CHX

Tests of Between-Subjects Effects

Dependent Variable: SQRTTR

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	157.107^a	7	22.444	14.710	.000
Intercept	1291.031	1	1291.031	846.145	.000
INFECT	123.400	1	123.400	80.877	.000
EXPTIME	2.644	1	2.644	1.733	.194
CHX	3.429	1	3.429	2.247	.141
INFECT * EXPTIME	16.948	1	16.948	11.108	.002
INFECT * CHX	5.473	1	5.473	3.587	.064
EXPTIME * CHX	8.524	1	8.524	5.587	.022
INFECT * EXPTIME * CHX	4.039	1	4.039	2.647	.110
Error	71.712	47	1.526		
Total	1462.021	55			
Corrected Total	228.818	54			

a. R Squared = .687 (Adjusted R Squared = .640)

Table 7. Tests of normality between infected and uninfected cells, cycloheximide treatment. Because there were less than 50 samples in each factor in the comparison, the Shapiro-Wilk test was used.

Tests of Normality

		Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	INFECT	Statistic	df	Sig.	Statistic	df	Sig.
SQRTTR	.00	.100	30	.200(*)	.957	30	.253
	1.00	.098	25	.200(*)	.968	25	.586

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Tests of Normality

		Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	EXPTIME	Statistic	df	Sig.	Statistic	df	Sig.
SQRTTR	short	.136	29	.181	.962	29	.373
	long	.146	26	.163	.942	26	.150

a Lilliefors Significance Correction

Tests of Normality

		Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	CHX	Statistic	df	Sig.	Statistic	df	Sig.
SQRTTR	no	.115	27	.200(*)	.945	27	.158
	yes	.170	28	.037	.929	28	.058

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

variable, while exposure time, cycloheximide treatment, and infection were used as fixed factors. Infection was used as fixed factors in this comparison because the comparison, in this case, was between either infected or uninfected cells. That is, unlike the previous comparison, which had tissues of different levels of infection, this comparison tested whether cells with the pathogen may become susceptible to cycloheximide treatment? The results indicated that infection was the only significant contributor to the variances in the percentages of apoptosis ($P<0.001$, $F=80.877$), while the two factors alone were not significant (time: $P=0.194$, $F=1.733$; cycloheximide: $P=0.141$, $F=2.247$). Also, two of the combinations of their interactions were significant (time X cycloheximide: $P=0.022$, $F=5.587$; time X infection: $P=0.002$, $F=11.108$), while the remaining combinations were insignificant (cycloheximide X infection: $P=0.064$, $F=3.587$; time X cycloheximide X infection: $P=0.110$; $F=2.647$) contributors to the variances in the percent apoptosis.

4.3.3 Staurosporine treatment

Brief exposure to staurosporine yielded the following results (Figure 17). In the case of brief exposure to this inducer, 9.77% of the cells in the uninfected samples were found to be apoptotic, while 88.78% of the cells in the uninfected samples were found to remain viable. In the infected samples briefly exposed with this inducer, 11.99% of the cells in these samples were found to be apoptotic while 86.32% of the cells in the infected samples remained viable. In the case of staurosporine-free controls, the uninfected samples had 10.59% of the cells undergoing apoptosis and 88.48% of the cells remaining viable. The remainder of the cells was either necrotic or dead. The

Effect of All Cells to Brief Exposure to STS

Figure 17. Comparison of percent apoptosis and viability among uninfected samples, infected samples, and infected cells in staurosporine (brief exposure treatment). Please note that uninfected samples contain only uninfected cells while infected samples contain both infected and uninfected cells. Infected cells contain only cells that were infected with *T. solenopsae*.

infected samples in staurosporine-free controls had 9.26% of the cells undergoing

apoptosis and 89.42% of the cells remaining viable. The remainder of the cells was

either necrotic or dead. In the case of infected cells alone, when briefly treated with

staurosporine, 32.32% of the cells were found to be apoptotic while 66.23% of the cells

remained viable. In the staurosporine-free controls, the infected cells had 23.49% of the

cells undergoing apoptosis while 75.84% of the cells remained viable.

Exposure to this inducer for one hour yielded the following results (Figure 18). 18.9%

of the cells of the infected samples were apoptotic when treated with this inducer while

79.61% of the cells of the infected samples remained viable. In the case of uninfected

samples, when treated with staurosporine for 1 hour, 22.56% of the cells became

apoptotic while 75.02% of the cells remained viable. In the staurosporine-free controls,

infected samples had 10.24% of the cells apoptotic, while 88.9% of the cells remained

viable. In the case of staurosporine-free, uninfected controls, these samples had 9.86%

of the cells apoptotic and 89.92% of the cells viable. In the case of infected cells,

treatment with staurosporine for 1 hour, 54.83% of the infected cells became apoptotic

while 44.58% of the infected cells in this treatment remained viable. Without

staurosporine, 30.07% of the infected cells became apoptotic while 67.72% of the cells

remained viable.

As in the analysis of the treatment by cycloheximide, two separate statistical tests

were performed. The first was a general linear model of ANOVA, which

comparedinfected and uninfected tissues, with the infected samples having various

Effect of All Cells to 1 Hr Expore of STS
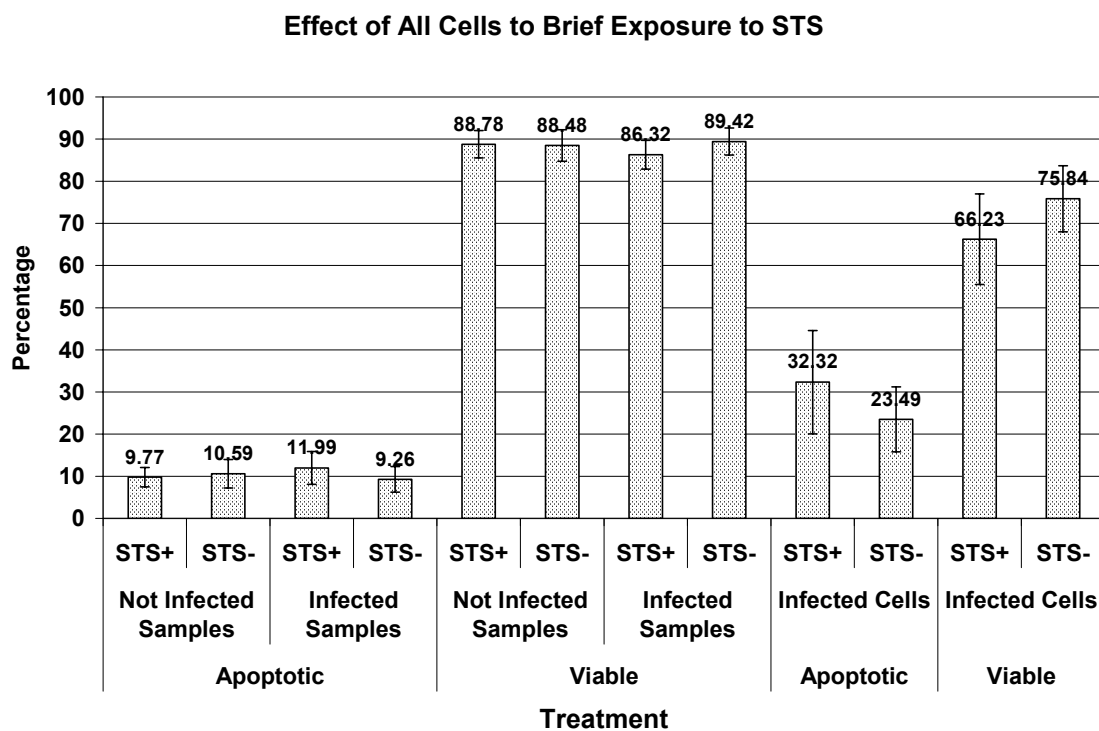

Figure 18. Comparison of percent apoptosis and viablility among uninfected samples, infected samples, and infected cells in staurosporine (1-hour exposure treatment). Please note that uninfected samples contain only uninfected cells while infected samples contain both infected and uninfected cells. Infected cells contain only cells that were infected with *T. solenopsae*.

percentages of infected cells in staurosporine treatment, both incubated for 1 hour and

brief exposure(Table 8). The dependent variable was the percent apoptosis while the

fixed factors included exposure time, infection state (infected or not infected), and

inducer treatment. The covariate was the percentage of infected cells. The data were not

transformed because they, according to the Shapiro-Wilk's test, do fit the normality

curve (Table 9). The ANOVA model also satisfied the equality of variances assumption

(Levene's test of equality of variances: P=0.673, F=0.699). The results of the analysis

showed that the percentage infected cells, the covariate, was not a significant contributor

to the variances of apoptosis (P=0.072, F=3.424) while exposure time and the

staurosporine treatment were significant (time: P=0.001, F=14.378; staurosporine:

P<0.001, F=20.806). The final fixed factor, the infection status, was not significant

(P=0.075, F=3.338). Only the interaction between time and inducer was significant

(P=0.002, F=11.228), while the remaining interactions were not (time X infection: P=0.3,

F=1.102; staurosporine X infection: P=0.815, F=0.055; time X staurosporine X infection:

P=0.059, F=3.783). As in the treatment with cycloheximide, a second test was

compared between infected and uninfected cells. However, the data was square root

transformed to fit with the normality curve (Shapiro-Wilk's test: Table 10). In this case,

the dependent variable was percent apoptosis, while the fixed factors included the

exposure time, inducer treatment, and infection. The test also satisfied the equivalence

of variance (Levene's test of equivalence of variance: P=0.129, F=1.731). The results

showed that all three fixed factors were significant contributors to the variances in:

Table 8. General linear model comparing infected and uninfected tissues in staurosporine treatment. INF: Infection status; INDUCER: Staurosporine; EXPOTIME: time of exposure to the Staurosporine; INFLEVEL: percentage of infected cells in infected tissues.

Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
INF	0	uninfected	24
	1	infected	24
INDUCER	0	no	24
	1	yes	24
EXPOTIME	0	brief	24
	1	1 hr exposure	24

Levene's Test of Equality of Error Variances[a]

Dependent Variable: PERAPOP

F	df1	df2	Sig.
.699	7	40	.673

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design:
   Intercept+INFLEVEL+INF+INDUCER+EXPOTIME+INF * INDUCER+INF * EXPOTIME+INDUCER * EXPOTIME+INF * INDUCER * EXPOTIME

Tests of Between-Subjects Effects

Dependent Variable: PERAPOP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1110.912[a]	8	138.864	7.575	.000
Intercept	1622.006	1	1622.006	88.480	.000
INFLEVEL	62.760	1	62.760	3.424	.072
INF	61.191	1	61.191	3.338	.075
INDUCER	381.420	1	381.420	20.806	.000
EXPOTIME	263.578	1	263.578	14.378	.001
INF * INDUCER	1.015	1	1.015	.055	.815
INF * EXPOTIME	20.208	1	20.208	1.102	.300
INDUCER * EXPOTIME	205.826	1	205.826	11.228	.002
INF * INDUCER * EXPOTIME	69.356	1	69.356	3.783	.059
Error	714.941	39	18.332		
Total	9808.126	48			
Corrected Total	1825.853	47			

a. R Squared = .608 (Adjusted R Squared = .528)

Table 9. Test of normality for the comparison between infected and uninfected tissue
samples when staurosporine was used as the apoptosis inducer. The Shapiro, Wilk's test
was used because the sample size was less than 50 in all comparisons.

Tests of Normality

	EXPOTIME	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for PERAPOP	low	.174	24	.057	.936	24	.133
	high	.132	24	.200(*)	.969	24	.637

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Tests of Normality

	INDUCER	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for PERAPOP	no	.086	24	.200(*)	.981	24	.914
	yes	.105	24	.200(*)	.976	24	.812

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Tests of Normality

	INF	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for PERAPOP	no	.117	24	.200(*)	.976	24	.815
	yes	.083	24	.200(*)	.986	24	.975

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Table 10. Tests of normality for the comparison between infected and uninfected cells when staurosporine was used as the apoptosis inducer. The Shapiro, Wilk's test was used because the sample size was less than 50 in all comparisons.

Tests of Normality

	INFLEVEL	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for SQRTAP	uninfected	.100	24	.200(*)	.957	24	.384
	infected	.152	24	.162	.952	24	.296

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Tests of Normality

	EXPTIME	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for SQRTAP	short	.126	24	.200(*)	.968	24	.621
	long	.098	24	.200(*)	.981	24	.919

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

Tests of Normality

	STS	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Standardized Residual for SQRTAP	no	.127	24	.200(*)	.966	24	.563
	yes	.079	24	.200(*)	.986	24	.972

* This is a lower bound of the true significance.
a Lilliefors Significance Correction

percent apoptosis (time P=0.001, F=12.573; staurosporine, P<0.001, F=16.158; infection: P<0.001, F=66.523). (Table 11) With regard to interactions, only the interactions between time and inducer was significant (P=0.008, F=7.687), while all other combinations of interactions were insignificant (time X infection: P=0.375, F=0.803; inducer X infection: P=0.268, F=1.263, time X infection X inducer: P=0.626, F=0.242).

4.4 Discussion

Pathogenic effects of intracellular protozoans on the host cells have only recently been investigated with regard to the effects on cell death (Heussler et al., 2001; Luder et al., 2001). In the first part of this study it was found that infected cells had higher percentages of apoptosis than uninfected cells, though the differences were not significant. Also, this part of the experiment showed that infected tissues had significantly higher percentage of apoptotic cells than uninfected tissues. Although the difference in the percentage apoptosis between infected and uninfected cells was not significant, but the low significance value (P=0.08) and that infected cells had in average higher percentages of apoptosis nevertheless suggested that the percentage of apoptotic cells among infected cells was higher than uninfected cells. Also, because in this part of experiment, no cells were found to be either necrotic (or late apoptotic) or dead, these data may suggest that the part of the apoptosis thus detected are more likely to be due to experimental handling of the tissues, rather that an event that is natural to the insect. This is particularly important because the method used to detect apoptosis here was the some percentages of cells should have gone into later stages of apoptosis; however, the

Table 11. General linear model ANOVA of staurosporine treatment at the cellular level.
INF: Infection status; EXPTIME: time of exposure; STS: treatment with staurosporine.
Univariate Analysis of Variance

Between-Subjects Factors

		Value Label	N
INF	0	no	24
	1	yes	24
EXPTIME	0	brief	24
	1	1 hr exposure	24
STS	0	no	24
	1	yes	24

Levene's Test of Equality of Error Variances[a]

Dependent Variable: SQRTAP

F	df1	df2	Sig.
1.731	7	40	.129

Tests the null hypothesis that the error variance of
the dependent variable is equal across groups.

a. Design: Intercept+INF+EXPTIME+STS+INF
 * EXPTIME+INF * STS+EXPTIME * STS+INF
 * EXPTIME * STS

Tests of Between-Subjects Effects

Dependent Variable: SQRTAP

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	95.162[a]	7	13.595	15.035	.000
Intercept	1029.573	1	1029.573	1138.699	.000
INF	60.148	1	60.148	66.523	.000
EXPTIME	11.368	1	11.368	12.573	.001
STS	14.609	1	14.609	16.158	.000
INF * EXPTIME	.726	1	.726	.803	.375
INF * STS	1.142	1	1.142	1.263	.268
EXPTIME * STS	6.950	1	6.950	7.687	.008
INF * EXPTIME * STS	.219	1	.219	.242	.626
Error	36.167	40	.904		
Total	1160.901	48			
Corrected Total	131.328	47			

a. R Squared = .725 (Adjusted R Squared = .676)

lack of necrotic and dead cells further suggest that part of the apoptosis were more likely due to experimental and manipulation error, as shown in the control samples. As a part of the procedure, internal organs such as the gut tissues, ovaries, poison glands, and spermatheca were removed prior to annexin-V staining and subsequent fixation in formalin. It is quite possible that some of these apoptotic cells had initiated apoptosis during and immediately after the organ removal. Also, these tissues are very closely associated with trachea and tracheoles. The removal of major organs prior to annexin-V staining may also cause at least some of the fat body cells to starve for oxygen, leading to apoptosis. However, these reasons only explained for the background apoptosis while they do not explain the significant difference in apoptosis in infected and uninfected cell in the treatments with apoptotic inducers.

When treated with cycloheximide, an apoptotic inducer that functions to block protein synthesis machinery of the target cell, it was found that, in the case of tissue comparisons, the only factor that showed any differences percent apoptotic cells was infection. That is, the differences in apoptosis between infected and uninfected tissues were significantly different; however, the percentage of infected cells was not a significant predictor of differences in apoptosis. Neither was the presence of the inducer a cause of any differences in apoptosis. These data suggested that the differences in apoptosis were not significant, other than the comparison of the types of tissues. Meanwhile, a comparison between infected and uninfected cells revealed that the only factor that showed the differences in apoptosis was the difference in cell type, be it infected or not, while the other fixed factors were insignificant. However, in the case of

interactions between factors, the interactions between time and cycloheximide, and time and infection were significant while the interaction between infection and cycloheximide was insignificant. These results indicated that there was a significant difference in apoptosis in infected vs. uninfected cells between the two time treatments, suggesting that there were higher percentages of infected cells in the incubated experiments. The results in the interaction of time X cycloheximide and time X infection and the insignificance of the cycloheximide X infection suggested that infection and cycloheximide worked separately with the time factor in the differences of apoptosis. Unfortunately, because this inducer failed to induce apoptosis in the *Thelohania*-negative controls in timed experiments, the data also suggested that the settings for this inducer were not optimized.

Thus, the fire ant fat body tissues were subjected to another apoptosis inducer. Treatment with staurosporine revealed the following patterns of apoptosis induction. The comparison between infected and uninfected tissues showed that the percentage of infected cells was not a significant predictor to the differences in percent apoptosis, while other fixed factors such as time of exposure and the presence of the inducer were. The only interaction term that was significant was between time and staurosporine, suggesting that staurosporine did in fact induce apoptosis in timed incubation. Because the interaction between time and staurosporine was significant, neither factor could be considered alone from the other. Thus, the conclusion that could be drawn in this comparison was that staurosporine caused apoptosis in timed experiment. When the comparison was made between the infected and uninfected cells, the results indicated

that staurosporine was effective at inducing apoptosis in both groups. The results further indicated that there significantly higher percentages of infected cells going into apoptosis than uninfected cells, and the results were not time and staurosporine dependent. This pattern was shown in the experiment that compared the fates of fat body cells without staining. Although the differences were not significant in that part of the experiment, the percentage of apoptotic cells was nevertheless higher in the infected cells than in the uninfected cells. However, only the interaction between time (brief exposure vs. one hour exposure) and the staurosporine components were significant, while other interaction components were not significant. Further, the finding that the interaction component between staurosporine and infection was not significant suggested that there might not be interactions between infection and staurosporine. However, because the underlying mechanisms of apoptosis in fire ants remains uninvestigated, more investigations to this issue is necessary to ascertain the interaction between the pathogen and the apoptosis induction. Thus, it can be concluded that at the tissue level, staurosporine was effective at inducing apoptosis; however, percent infection was not a significant predictor of percent apoptosis. At the cellular level, it can be concluded that the difference in percent apoptosis could be explained significantly by infection. In other words, at the cellular level, there was significantly higher percentage of apoptotic cells in the infected cells than in the controls. Also, staurosporine and time together were significant contributors of apoptosis. Because the interaction component between staurosporine and infection was not significant, it can be concluded that staurosporine did not cause any significant differences in apoptosis between infected and uninfected

cells, although it did cause significant increase of percent apoptosis in the two different time frames.

The results of the staurorporine experiment showed that there were significantly higher percentages of infected fat body cells undergoing apoptosis than uninfected fat body cells. Although the first experiment on the direct observation without any inducer show insignificant difference with the average percentage of apoptosis higher in the infected cells than uninfected cells, the two latter experiments clearly demonstrated that infected cells had significantly higher percentages of apoptosis than uninfected cells. Also these results suggested that the infected cells were not resistant to apoptotic insults. Intracellular protozoan pathogens have been found to cause their host cells to be either promoting or resisting apoptosis, most of which have been done with vertebrates and their intracellular protozoan pathogens (Heussler, 2001; Luder et al., 2001). *Toxoplasma gondii*, for example, has an elaborate mechanism to inhibit programmed cell death (Sinai et al., 2004). However, in the case of ocular taxoplasmosis in mice, Fas and FasL were found to be up regulated in infection, inducing apoptosis (Hu et al., 1999). In the case of malaria pathogen *Plasmodium falciparum* infection of vertebrate erythrocytes, the host cells have evolved defenses against this pathogen by allowing the externalization of phosphatidylserine and the subsequent apoptosis of the infected cells, allowing the infected cells to be phagocytized (Lang et al., 2004). In the case of infection with *Cryptosporidium parvum*, Elliot and Clark (2003) found that the cells from MDCK cell lines would undergo necrosis instead of apoptosis. Finally, another microsporidium, *N.*

algerae, was shown to cause the infected cells to become more resistant to apoptotic insults (Scanlon et al., 1999).

Important differences exist between the experiments here and the experiments of other intracellular protozoan pathogens such as Scanlon et al., (1999) and the experiments cited by Heussler (2001) and Luder et al., (2001). Whereas Scanlon et al. (1999) used cell lines, the experiment here used freshly extracted fire ant tissues. Whereas Scanlon et al. (1999) were able to artificially inoculate cells with *N. algerae,* reliable methods to artificially inoculate fire ant cells with purified forms of viable *Thelohania* spores do not exist. Therefore, the experiments here were limited to use only already infected samples and cells. The results presented here may further be verified whenever there are major breakthroughs in the culturing of fire ant cells and the ability to artificially infect cultured fire ant cells with viable, purified *T. solenopsae* spores. The experiment here found that the percentage of apoptosis between infected cells and uninfected cells was significantly different. Unfortunately, induction of apoptosis by *T. solenopsae* was not part of this experiment due to the lack of suitable technologies. Nevertheless, the finding that the percentage of apoptotic, uninfected was different from the percentage of apoptotic, infected cells should predict that infection by *T. solenopsae* may induce apoptosis. Further experiments, along with suitable techniques, will be needed to verify this.

The findings here showed that there were significantly more apoptotic cells in infected cells than in control cells. These findings predict the induction of apoptosis of host cells by *T. solenopsae,* while the data here do not support previous findings that

infected cells may become resistant to apoptotic insults (Heussler et al., 2001). Whereas

other intracellular protozoan pathogens are known to suppress apoptosis via factors such

as NFκB and HSP70 (Heussler et al., 2001), the results here may not support the

activation of these genes in fire ants. More research, including the identification of

NFκB and HSP70 in fire ants would be necessary to show this.   Pro-apoptotic factors

such as Fas and FasL are known to be up-regulated and apoptosis was observed in ocular

taxoplasmosis in mice (Hu et al., 1999), the results here may predict the activation of

these genes. Another possibility may be something similar to the apoptotic effects of *C.

parvum* in the intestinal and binary epithelial cells. In this case, *C. parvum* was found to

induce apoptosis in the binary and intestinal cells (Chen et al., 1999). Whereas *C.

parvum* was found to directly induce apoptosis, the data here predict the notion of

apoptosis induction in infected cells. Chen et al., (1999) showed that apoptosis is

triggered by a Fas – ligand dependent mechanism. Again, more research into the

molecular mechanisms of apoptosis induction in fire ants would be needed to confirm

this.

CHAPTER V

CONCLUSIONS

Thelohania solenopsae is a eukaryotic, intracellular pathogen that belongs to the phylum microspora. *T. solenopsae* is known to infect both species of the imported fire ants, *Solenopsis invicta* and *S. richteri*. These pathogens were first discovered from preserved fire ant specimens from Brazil (Knell et al., 1977). These pathogens were not discovered in North American populations of the red imported fire ants until 1998 (Williams et al., 1998). Since their discovery, various aspects of the biology of the pathogen had been investigated. For example, a basic picture of the life stages were investigated by Knell et al., (1977), which found at least two forms of spores in adult ant, the octospores and the free spores. Further investigations by Sokolova and Fuxa (2001) and Williams et al., (2001) revealed that there were more than three spore types. Molecular approaches studying the relationship between North and South American species of *T. solenopsae* were investigated by Moser et al., (1998; 2000), which suggested that, the rDNA genes between North and South American populations of *T. solenopsae* were different in several nucleotides, but not different enough to be considered as distinct species. Studies on the effects of this pathogen to their fire ant hosts had been studied. For example, Briano et al., (1995a) found that infected colonies had a significantly smaller mound volume than uninfected colonies. Also, Williams et al., (1999) found that, 23 – 29 weeks after inoculation with *T. solenopsae*, there was at least an 88% decline in the brood in the fire ant colonies. Transmission studies had been

done by Oi et al., (2001). In that study, it was found that workers were required to achieve transmission of *T. solenopsae* from brood to an uninfected queen.

Although much had been done in the research of microsporidiosis in the red imported fire ants, three areas of the association between this pathogen and the red imported fire ant were nevertheless identified as components of this project, including horizontal transmission of the pathogen, pathogenic effect on humoral proteins of the fire ant host, and effects on apoptosis of host cells. Horizontal transmission chapter focused on the possible contamination of exudates from the insect hosts, the following were investigated: adult secretions, larval secretions, and midden piles. The first objective of the first component focused on the adult secretions and midden piles while the second objective focused on the larval secretions. For the hemolymph proteins, the differences in the profiles of total hemolymph proteins among infected and uninfected queens, which included alates, inseminated dealates, and uninseminated dealates, were compared. Also, the titers of a major hemolymph protein, vitellogenin, were compared between infected and infected dealates. In the investigations of induction of apoptosis, fire ant fat body tissues were investigated for signs of apoptosis without any incubation and without any apoptosis inducers. This was done to examine whether or not the pathogen induces apoptosis in their host cells. In the second objective, fat body tissues from both infected and uninfected inseminated dealates were treated with two inducers, cycloheximide, which induces apoptosis by inhibiting protein synthesis. Finally, effects of another apoptosis inducer, staurosporine, a protein kinase C inhibitor, were investigated and compared between infected and uninfected fire ant inseminated dealates.

The horizontal transmission scenario for *Thelohania solenopsae* in fire ants remains uncertain. In the first objective, secretions such as anal fluid and crop fluid from adults were investigated for the possible contamination of spores. It was discovered that a majority of the fecal fluid samples tested contained spores, suggesting that this may be the possible source of spores in an adult-only transmission scenario. Although some spores were found in the crop fluid, the presence of the buccal filter in workers, which do not permit the passage of particles larger than 0.88 μm in size, make it an unlikely source of spores. However, whether this blockage is unidirectional or bidirectional remains to be tested. The source of the spores in the crop fluid is also unclear. The lack of spores in hemolymph samples suggested that the spores found in both the anal and crop fluids were unlikely to be from hemolymph contamination. Because spores are much larger than the size of the filter, spores may not be swallowed by the adult workers. Whether or not the unswallowed spores are stored in the buccal cavity still remains to be shown. However, larvae and possibly queens do not possess this buccal filter and thus may swallow and become infected with the pathogen. If the workers do indeed store the spores in the buccal cavity, this may further support the theory that workers, while imbibing the meconia materials from pupating, infected larvae, store the spores in the crop and regurgitate the spores to other members of the colony. This scenario would further provide mechanistic explanation of the findings of Oi et al., (2001). Finally, the presence of spores in the midden piles may suggest the possibility of environmental contamination by the spores. This may serve as a mean of dissemination of spores into the environment.

Larval secretions examined for the presence of *T. solenopsae* spores include oral fluid, anal fluid, midgut, meconium, and pupal exuviae. In these samples, only the midgut and the meconium contained spores. Therefore, the transmission scenario *T. solenopsae*, is most likely through the meconium. Although a direct feeding experiment using meconium was not part of this study, the finding that workers extract and share the meconium from pupating larvae (Cassill and Vinson, unpubl.) provides strong evidence that *T. solenopsae* achieves horizontal transmission through meconia. This transmission scenario may explain both intracolonial and, to some extent, intercolonial transmission. In the case of intercolonial transmission, this might take place during brood raiding (Williams et al., 1999; Oi and Williams, 2002).

Currently, the only known method to reliably inoculate fire ant colonies with this pathogen is by using brood from infected colonies. This finding provides a proximate explanation to the infection of colonies using infected brood. Previously, it was known that using infected brood is effective at inoculating fire ant colonies. The findings here provided a mechanistic explanation for how this may work. After infected brood was placed into target colonies, workers will adopt them as their own. When the infected brood pupates, the workers would remove the infected meconia and share the materials with the rest of the colony, thus infecting other nestmates.

Further experiments must be done to further characterize horizontal transmission of this pathogen in the fire ants. Although meconium was shown to be contaminated with *T. solenopsae* spores, whether or not meconia were indeed infectious was unknown. Feeding experiments using extracted meconia from infected colonies will further

elucidate the utility of these exudates from pupating larvae in the transmission of this pathogen. Also, examination of buccal cavity of infected workers will explain whether the spores from meconia are stored in the buccal cavity to be fed to the remainder to the colony. Because midden piles were shown to harbor spores, experiments may be performed to examine the infectivity of the infected midden piles. For example, midden piles from infected colonies could be transferred to uninfected colonies to examine whether or not the uninfected colonies may be infected this way. Another possibility is to verify importance of environmental contamination by rearing known uninfected colonies in the nest soil of the known infected colonies. The uninfected colonies may then be examined at various time intervals in terms of weeks for signs of infection. The controls may include rearing uninfected colonies in the soil from which uninfected colonies are obtained.

In the second section, the effects of *T. solenopsae* infection on humoral proteins were investigated because the target cells of infection by this pathogen are fat body cells (Knell et al., 1977), which are major contributors to the humoral proteins (Keeley 1985). SDS-PAGE gels were run on three types of queens, either infected or uninfected: alates, inseminated dealates, and inseminated dealates. In the first analysis, it was found that infected queens, in all three classes, had significantly fewer protein bands than their uninfected counterparts. This suggested that the infection with this pathogen altered the hemolymph physiology of the host. These data suggested that infection by this pathogen did not lead to the activation of an immune cascade because if immune cascade were activated, then there should be more protein bands in the infected samples than in the

uninfected samples. This might be the case due to their intracellular nature of parasitism, as part of the data suggested that no spores were found in the hemolymph. This is congruent with findings in other microsporidiosis. For example, Becnel and Andreadis (1999) suggest, immune responses are usually not triggered unless the integrity of host cells were compromised. A further comparison revealed that different protein bands were down regulated in different types of infected queens. This finding did follow the findings that different queen types have different humoral physiology (Lewis et al., 2001, Consoli and Vinson, 2002). However, the mechanisms that lead to such differences in humoral physiology due to infection still remained to be investigated.

The differences in titers of vitellogenin were examined between infected and uninfected dealates. The data suggested that there is a drop in the titers of this protein in infected samples. This finding was congruent with other findings. Hurd (2001) showed that the fecundity of *T. molitor* was reduced following tapeworm infection and that this reduction was correlated with a reduction in vitellogenin in the hemolymph. Hurd and Webb (1997) argued that the tapeworm caused a reduction of host fecundity because the tapeworm utilized the nutrients that the host otherwise needed for egg production. This line of reasoning could also be true in the case of *T. solenopsae* infection in fire ants. Another factor that may have caused the decline of vitellogenin might be the disruption of the fat body cells by *T. solenopsae*, assuming that structurally damaged cells could not produce proteins as well as uninfected cells. Although details of the disruption of the fat body cells in the case of infection by this pathogen has not been shown, the findings of the infected cells by other species of microsporidia suggest a severe

disruption of the cells the pathogen inhibits. For example, in *T. tipulae* infected fat body cells from larval *Tipula literalis*, the pathogen replaced all of cytoplasmic organelles except the nucleus and mitochondria (Weissenberg, 1976, Tanada and Kaya, 1993; Becnel and Andreadis, 1999). Although infected cells are affected, the finding that only approximately 16% of the fat body cells were infected suggested that the alteration of humoral proteins may not be caused only by the infected cells. In fact, it is possible that the reduction in vitellogenin and the reduction of humoral proteins could be a global response to infection by this pathogen. If the reduction in vitellogenin in infected fire ants were caused by the infected cells alone, then the reduction should not be this drastic. The exact mechanism of vitellogenin reduction in the infection of *T. solenopsae* still remains to be investigated.

One of the major problems with using SDS-PAGE was of its low resolution. Each band on an SDS-PAGE gel may represent several proteins of the same molecular weight. Therefore, instead of using SDS-PAGE, two-dimensional gel electrophoresis may be utilized to give higher resolution to differences between different types of queens. By using this technology, differences at the level of individual proteins could be resolved. Further, the different proteins may be isolated and sequenced and their functions may then be identified by comparing the sequences of these proteins with the known proteins that have similar primary structure. Another possibility would be to include males into the studies to determine any sex-specific changes in the humoral proteins due to infection of this disease. This may either be done using SDS-PAGE or for better resolution, using 2-D gel electrophoresis. A major problem with the project

was that the age of queens and the length of time they had the disease were unknown. An improved version of the experiment would involve artificially inseminating laboratory-reared alates of known age, calculated as time after eclosion. The artificially inseminated queens would then be artificially inoculated with *T. solenopsae* by providing her with infected brood and workers. Hemolymph samples from queens of different age may then be analyzed. The controls would involve mock inoculation using brood from known uninfected colonies. Unfortunately, this experiment cannot be done as yet because no method is currently known to artificially inseminate a fire ant queen.

The final component of the pathology investigated was whether infection would be related to apoptosis of the host. It was found that there was significant difference between the percentage of apoptotic cells between infected and uninfected tissues. A further investigation comparing the infected cells found in the infected tissues (infected tissues contained both infected and uninfected cells) and uninfected cells from uninfected tissues (uninfected tissues had only uninfected cells) revealed that the percent apoptosis in infected cells was not significantly different from the percent apoptosis of uninfected cells. However, because the average of apoptotic infected cells were higher than apoptotic, uninfected cells and because the P value was 0.08, it was concluded that, although the differences were not significant, there were nevertheless higher percentages of infected apoptotic cells than uninfected apoptotic cells. This was confirmed by later experiments using inducers of apoptosis. These data suggested that infected cells had significantly higher percentage of apoptotic cell than uninfected cells.

Two different inducers of apoptosis were used in the next experiment to examine their effects on apoptosis in *T. solenopsae*-infected tissues and cells. The first inducer used was cycloheximide, which induces cell death by inhibiting protein synthesis. In this experiment, fat body tissues from both infected and infected fire ant inseminated dealates were either treated not treated with this inducer. The two time frames compared tissues which were briefly treated with the inducer, and tissues which were incubated with cycloheximide for four hours. The results, analyzed by using a general linear model ANOVA, found that the inducer did not stimulate apoptosis of the tissues under those experimental conditions. When compared between infected and uninfected cells, it as found that the differences in apoptosis were due to either the interaction between time and cycloheximide, or between time and the pathogen. Thus, it was concluded that, in the treatment with cycloheximide, the inducer did in fact induce apoptosis and there were significantly more infected apoptotic cells than uninfected apoptotic cells and the was no evidence that infected cells became resistant to apoptotic insults. Unfortunately, because the uninfected controls failed to show apoptosis in timed experiment with cycloheximide as the inducer, it was concluded that the experimental conditions were not optimized for cycloheximide assay.

The other inducer tested was staurosporine. The comparison between infected and uninfected tissues revealed that the percentage of infected cells was not significant in predicting the differences of apoptosis between the tissue types. The significant predictors included interaction of time of exposure and the inducer, suggesting that the staurosporine induced apoptosis detectable at the tissue level. The comparison at the

cellular level showed that there were significantly higher percentages of apoptotic cells in the longer time treatment with the inducer in the case of infected cells than uninfected cells. The results further showed that staurosporine did cause apoptosis in all cells treated. Also, there was a difference in apoptosis between infected and uninfected cells. Because the interaction between staurosporine and infection components was not significant while infection by itself was significant, it was concluded that infection was correlated to higher percentages of apoptosis and the inducer did induce apoptosis in all cells. Finally, there was no evidence that infected cells became resistant to apoptotic inducers.

In this project, the presence of the pathogen could not be used as a causation to apoptosis even if differences existed because currently there are no known methods to directly inoculate purified *T. solenopsae* spores into fat body tissues. Also, no known cell lines exist for *S. invicta* (Consoli, pers. Comm.). As a result, causation cannot be proven until suitable methods are developed to inoculate *T. solenopsae* spores into fire ant cell lines, as in the case with the investigations with other intracellular protozoan pathogens (Heussler et al., 2001; Luder et al., 2001).   The microsporidia, *Nosema algerae*, is the only other known species of microsporidia whose effects on host cells have been investigated (Scanlon et al, 1999).

Further experiments may be done better characterize the correlation between apoptosis and infection by *T. solenopsae*. Because this project had shown that infection was correlated with significantly higher percentages of apoptotic cells, a comparison on the level of DNA fragmentation could be performed because DNA fragmentation is one

of the major characteristics of apoptosis. To understand further the pathways of apoptosis in fire ants, major genes such as *Bcl*-2 and caspases that control apoptosis must be identified. This can be achieved by first designing degenerate primers based on the known sequence of the gene. The genes can be identified by applying polymerase chain reactions based on the degenerate primers. The experiments in this project only demonstrated apoptosis in the aspect of phosphatidylserine externalization. Other aspects of cell death, such as fragmentation of DNA, can be demonstrated using the TUNEL assay (Silva et al., 2000).

In conclusion, there were three major findings in this project. First, meconium was found to be the most likely candidate responsible for horizontal transmission of *T. solenopsae* in *S. invicta*. It contained spores and the materials were shared with other nestmates via other adult workers, as described by Oi et al. (2001). Secondly, differences were detected between the banding patterns of the total proteins in the hemolymph samples among three types of fire ant queens and vitellogenin was found to be significantly reduced in the infected dealates. The finding that infected cells were less than 20% of the total cells suggested that the reduction in vitellogenin in the hemolymph in infected fire ant queens may be a systemic effect, rather than localized to only infected cells. Finally, the correlation between infection and apoptosis by was investigated. It was determined that infection was correlated to higher levels of infection and that cycloheximide was not optimized while staurosporine was optimized to induce apoptosis. There was no evidence to supports that infected cells become resistant to apoptotic inducers.

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APPENDIX

FORMULA FOR THE FIRE INCUBATION MEDIUM

COMPONENT	g/500ml
AMINO ACIDS: L-FORM	
Alanine	0.062
Asparagine	0.018
Arginine	0.028
Apspartic Acid	0.001
Cysteine	0.001
Glutamic Acid	0.024
Glutamine	0.030
Glycine	0.094
Histidine	0.072
Isoleucine	0.040
Leucine	0.108
Lysine	0.072
Methionine	0.028
Phenylalanine	0.075
Proline	0.611
Serine	0.032
Threonine	0.032
Tryptophan	0.015
Tyrosine	0.190
Valine	0.085
SALTS	
NaCl	4.588
KCl	0.298
$CaCl_2$(anhydrous)	0.222
OR	
$CaCl_2$(dehydrate)	0.294
$MgSO_4$	0.301
BUFFER	
HEPES	2.980
MISCELLANEOUS	
Glucose	0.500
Trehalose	5.000

Adjust to pH 7.2 with NaOH.
Sterile filter.

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