INTERACTIONS BETWEEN *FRANKLINIELLA FUSCA* (THYSANOPTERA: THRIPIDAE),
*THRIPINEMA FUCSUM* (TYLENCHIDA: ALLANTONEMATIDAE), AND
ENTOMOPARASITES (*TOMATO SPOTTED WILT VIRUS*, *PANTOEA ANANATIS*, AND
*WOLBACHIA*)

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010
To my family Ford Sims, Sandy Morgan, Laura Bonsky, Olga Kapps, and especially James C. Dunford, Kia, Willie and Ziggy for their unconditional love and support while I pursued my entomological studies, making this milestone possible.
ACKNOWLEDGMENTS

I would first like to thank my advisors Joe Funderburk (University of Florida’s North Florida Research and Education Center in Quincy, FL [UF-NFREC]) and Drion Boucias (UF Department of Entomology and Nematology). Their advice, patience, guidance, and friendship have made this research possible. I would also like to thank my committee members Stuart Reitz (United States Department of Agriculture - Agricultural Research Service - Center for Medical, Agricultural, and Veterinary Entomology in Tallahassee, FL [USDA-ARS-CMAVE]), Jimmy Becnel (USDA-ARS-CMAVE in Gainesville, FL) and Timur Momol (UF-NFREC in Quincy, FL) for providing their time and expertise. Most of this research would not have been possible without their help.

A very special thanks goes to the numerous individuals who have provided knowledge, equipment, supervision, and expertise on topics covered in this project: Byron Adams (Brigham Young University), Kostas Bourtzis (University of Ioannina), James Boyer (UF’s Plant Science Research and Education Unit in Citra, FL), Ellen Dickstein (UF’s Plant Pathology), Karen and Kim Kelley (UF’s Interdisciplinary Center for Biotechnology Research [UF-ICBR]), Dean Paini (UF-NFREC), Dorith Rotenberg (Kansas State University), Savita Shanker (UF-ICBR), Mitrinjai Srivistava (UF-NFREC), Julie Stavisky (UF-NFREC), Michelle Stuckey (UF-NFREC), Chris Tipping (UF-NFREC), Carl Vining (UF-PSREU), Anne Whitfield (University of Wisconsin), and Donna Williams (UF-ICBR).

I would like to thank numerous faculty, staff and students at the University of Florida’s Department of Entomology and Nematology for making my research a rewarding and fulfilling experience: Carl Barfield, Marc Branham, Lyle Buss, John Capinera, John Denton, Don Dickson, Dan Fitspatrick, Judy Gillmore, Maria Gomez, Debbie Hall, Don Hall, Amanda Hodges, Nick Hostettler, Pam Howell, Marjorie Hoy, Steve Lasley, Verena Leitze, Norm
Leppla, Oscar Liburd, Ale and Jim Maruniak, Heather McAuslane, Jane Medley, James Nation, Khuong Nguyen, Linda Pedersen, Pannipa Prompiboon, Tamer Salem, Nancy Sanders, Mike Scharf, Grover Smart, Matt Tarver, and Mary Kay Weigel. A very special thank you goes to those who contributed to my teaching experience at the University of Florida; they are: Doug Levey, Elisa Livengood, Suzan Smith, Carmella O’Steen, and Dale Witt. I would also like to thank those who opened their hearts and their homes to me: Judy Boucias, Lincoln Brower, and Miriam Funderburk.

Most importantly, I would like to thank my family and friends for their support and patience: Bob and Maude Ashley; Marcia and Paul Blankenship; Laura, Michael, Mikayla, Taryn and Ryan Bonsky and Aaron Salazar; Brent Brooks; Karen Buchanan and her family; Nicole, Andrew and Brooklyn Butler; Glen Cannon and Jean Thomas; Becky, Carl and Amanda Carter; Frank and Mary Cassett; Karen and Alyssa Christner; Adie Davies; Jim, Kia, Willie, and Ziggy Dunford; James F. Dunford; Kim Dunford; Kathy and Vance Eaddy; Imogene Floyd; Evelyn Green and Joe Hopkins; Marie Hershberger; Carol Kapps; Olga Kapps; Kat and Bennett King; Lynne and Greg McCrae; Jennifer and Jason Meyer; Sandy and Mike Morgan; George, Dina and Caroline Nunn; Lisa Nunn, Pat Fry and Frankie DeAngelis; Gale and Jim Owens and their family, Heather and Emmalee Larsen, Rachel, Jeff, Caitlyn and Lily Thomas and Jamie, Kelly and Erin Owens and Natalie Shaw; Andrea, Matt and Taryn Pawl; Kitty Phelps; John Ridgway; Betty Romano; Fran, Pete and Francine Rossi and Carol Field; Carolyn Sabol and her family; Karen and Lee Schwind; Ford Sims; Teri Stuber; Glenn and Joan Taylor; April Terry; BJ Thornton; Margo and Roger Thrall; Laurie and Calvin Trenholm; and Joanne Wilson.
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By

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August 2010

Chair: Joseph Funderburk
Major: Entomology and Nematology

*Frankliniella fusca* (Hinds) (Thysanoptera: Thripidae) is an important vector of bacterial and viral plant pathogens in various cropping systems. The entomogenous nematode *Thripinema fuscum* Tipping & Nguyen (Tylenchida: Allantonematidae), a natural enemy of *F. fusca*, is a key regulator of *F. fusca* populations and the diseases they vector. The obligate nematode renders host female thrips sterile and reduces secondary disease spread. The data presented here suggest *Thripinema* parasites also reduce primary disease spread by modulating the density of entomoparasites within their thrips host. The overall objectives of this research were to (1) identify entomoparasites associated with *F. fusca* using a combination of histological, molecular, and biological techniques and (2) determine if, and to what extent, the obligate parasite *T. fuscum* influences the biology of *F. fusca* and its association with entomoparasites (*Wolbachia, Pantocea ananatis*, and *Tomato spotted wilt virus*). Herein, I provide a detailed histological examination documenting the internal morphology of healthy *F. fusca* females, the life cycle of *T. fuscum*, and identify changes to thrips target tissues and cells resulting from *T. fuscum* parasitism. I also report that *F. fusca* and *T. fuscum* are infected by the reproductive parasite *Wolbachia*, and that parasitism by *T. fuscum* induces a switch in supergroup designation of the dominant *Wolbachia*
strain present in host thrips. In addition, I document that parasitism by *T. fuscum* reduces the feeding rates of *F. fusca* females by 65% and TSWV transmission by 50%. Lastly, I demonstrate *T. fuscum* parasitism reduces both the incidence of *F. fusca* individuals harboring the plant pathogens *P. ananatis* and TSWV and the pathogen titers in infected individuals. Results from this project suggest that the response of a host to a parasite/pathogen is dependent upon its interaction with other microorganisms. From a broader perspective, the information generated by these studies may provide a better understanding of how parasites interface with entomogenous agents in their insect vectors. Understanding how these interactions influence vector competence may one day provide targets for suppressing disease spread and provide a unique complement to existing disease management strategies for controlling thrips vector populations.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW: RESEARCH BACKGROUND, OBJECTIVES, AND HYPOTHESES

Introduction and Literature Review

The following literature review provides an overview of each organism involved in this multitrophic system, and addresses the most important aspects of each as they pertain to my research topic. I have organized the literature review into three main sections: (1) Thysanoptera, (2) Entomoparasites of *Frankliniella fusca*, and (3) Objectives, Hypotheses, Goals and Expected Outcomes. There are numerous cross-references throughout the dissertation which help to integrate the various topics as they refer to *Frankliniella fusca*.

Thysanoptera

Natural history of thrips

The evolutionary history for Thysanoptera, or thrips (pl. and singl.), dates back 230 million years with the recent world species dominated by taxa that evolved within the last 40-50 million years (Grimalidi et al., 2004). Thysanoptera presumably shared a common ancestor with Hemiptera (true bugs), Psocoptera (booklice), and Phthiraptera (sucking lice) based on homology of mouthparts, antennal sense cones, absence of ocelli in larvae, and reduction in Malpighian tubules; these four orders currently comprise the hemipteroid insect lineage (Ananthakrishnan, 1979; Mound, 1997; Gillot, 2005). Primitive thrips species fed on liquid from decaying tissues and fungal hyphae that were often associated with unstable microhabitats (Mound, 1997). The ability of ancestral thrips to exploit ephemeral and optimal conditions facilitated the development of several “r-selected” biological characteristics including polyphagy, vagility, short generation time, tendency towards parthenogenesis, and a competitive breeding structure promoting aggregation and exploitation of localized optimal conditions (Mound, 1997). Over time, these traits predisposed the Thysanoptera to become opportunistic species successful in exploiting a
variety of habitats in tropical, subtropical, and temperate regions, and many thrips species are now adapted to an invasive lifestyle (Ananthakrishnan, 1984; Mound, 1997; Morse and Hoddle, 2006). In recent years, human transport has played a significant role in the establishment of thrips populations in non-native habitats (Mound, 1983; Kirk and Terry, 2003). To date, thrips are cosmopolitan in distribution and numerous food, fiber, and ornamental crops serve as habitats for aggregating populations (Lewis, 1997a; Mound, 1983, 2005).

Thrips were first described by DeGeer in 1744 as *Physapus*, but were later moved to the genus *Thrips* by Linnaeus in 1758 (Lewis, 1997b). In 1836, *Thrips* was raised to the order Thysanoptera by Haliday. The etymology of the word Thysanoptera comes from the Greek words “thysanos”, a fringe, and “pteron”, a wing. There are currently over 5,500 species of thrips described, and Mound (1996) speculates this number represents only 60% of total thrips species in existence. Thrips are divided into two suborders, the Tubulifera and Terebrantia. This division is based primarily on the shape of abdomen and ovipositor, although wing characteristics and number of larval stages are also used to distinguish the suborders. Tubulifera have the last abdominal segment modified into a tube-like structure used for depositing eggs on vegetation. This group includes over 3,100 species (known from one family, Phlaeothripidae) that feed on a wide range of resources including fungal spores or hyphae, mosses, tropical trees and shrubs, flowers, mites, lepidopteran eggs, scales, and whiteflies (Mound, 1997). The remaining seven Thysanopteran families belong to the suborder Terebrantia. This suborder is distinguished from Tubuliferan thrips by the presence of a saw-like ovipositor on the ventral side of the last abdominal segment that is used for inserting eggs into host plant tissue. The vast majority of thrips within Terebrantia feed on leaves, flowers, pollen and grasses, and many are considered crop pests. The most economically important crop pests in the Terebrantia belong to
the family Thripidae (*Thrips* and *Frankliniella* spp.) and include over 2,000 of the 5,500 described thrips species (Mound, 1997, 2002).

Thysanoptera are a remarkable group of insects with several defining biological characteristics. Thrips have an unusual development, often described as intermediate between holo- (complete) and hemi- (incomplete) metabolous, in which there are two active larval stages and two to three quiescent pupal stages prior to adult emergence (Takahashi, 1921). Morphologically, thrips are the only insects that possess asymmetrical hypognathous mouthparts. The right mandible is resorbed during embryonic development and the single left mandible and two developed maxillae are reduced to an asymmetrical stylet used for ‘punching and sucking’ cell contents (Chisholm and Lewis, 1984; Heming, 1978, 1993; Moritz, 1997). Other defining characters include a protrusible arolium (or bladder) on the pretarsal end that can be everted by hemolymph pressure to allow thrips to walk vertically on surfaces (Heming, 1971). When present, wings are linear and fringed with long marginal setae that function as slots to increase wing surface area and allow individuals to travel aerially over long distances (Lewis, 1997). Thrips are polymorphic in wing formation and can develop into macropterous (wings present), micropterous or brachypterous (wings reduced to wing pads), or apterous (wingless) morphs (Mound, 2005). Wingless species, although not able to fly, are capable of aerial dispersion and some species have naturally dispersed over 1,000 miles (Mound, 1983). The mechanisms determining wing form are not well understood but are thought to be the result of both genetic and environmental factors such as photoperiod, crowding, and food limitation (Hood, 1940; Koppa, 1970; Kamm, 1972; Zera and Denno, 1997; Mound, 2005).

The small size (~1-5 mm) and thigmotactic behavior of thrips enables species to occupy diverse vegetative microhabitats and populations can be (but are not restricted to) anthophilous
(flower-inhabiting), phyllophilous (leaf-inhabiting), poephilous (grass-inhabiting), phlaeophilous (bark-inhabiting), or cecidicolous (gall-inhabiting) (Ananthakrishnan, 1984; Moritz, 1997; Sabelis and Van Rijn, 1997). Two thrips species are sub aquatic and reside in slime within the stalks and reeds of water plants (Lewis, 1973). The ability of thrips to occupy a wide variety of niches has resulted in the development of many diverse polymorphisms and behaviors (Moritz, 1997; Mound, 2005; Morse and Hoddle, 2006). Thrips can be phytophagous (tissue and pollen feeders), fungivorous (mycophagous or sporophagous), predacious (on mites, aphid and whiteflies), omnivorous, cannibalistic, and ectoparasitic (Darwin, 1876; Ananthakrishnan, 1984; Izzo et al., 2002; Terry, 1997, 2001) (Figure 1-1). Thrips behavior involves a broad range of visual ecological cues, host odors, and semiochemicals (Terry, 1997). Many thrips species display sexual dimorphism, with males possessing modifications in body form, cuticular structure, color, setae and genitalia (e.g., Mound et al., 1998; Mound, 2005; Tyagi et al., 2008). Thrips that do not display apparent sexual dimorphism compensate by possessing unique behavioral traits (Crespi, 1988, 1990). In one of the most extreme cases of dimorphism within the order, some gall-forming species exhibit territoriality with sophisticated social behavior including the development of soldiers with reduced reproductive potential that suggests the evolution of eusociality (Kirk, 1985; Crespi, 1992; Gillespie et al., 2002; Mound, 2005).

Sexual reproduction is the primary reproductive strategy in the haplodiploid Thysanoptera. However, many phytophagous thrips also exhibit arrhenotokous (unfertilized eggs develop into males), thelytokous (unfertilized eggs develop into females), or deuterotokous (unfertilized eggs develop into both genders) parthenogenesis (Moritz, 1997). In some thrips species, sex determination may be microbe-associated (Kumm and Moritz, 2008; See Chapter 2). The number of offspring produced by a female varies upon dietary and environmental conditions, but
most females are highly fecund and oviposit between 30 to 300 eggs over their lifetime (Lewis, 1997). A typical life cycle is completed in two to three weeks and the number of generations per year ranges from one to 15, the latter has species that are often multivoltine (Lewis, 1997). Most thrips field populations are bisexual with sex ratios being primarily female-biased (Higgins and Myers, 1992; Tsuchida and Ohguchi, 1998; Hoddle, 2002; Reitz, 2002; Vasiliu-Oromulu, 2002; Sims et al., 2005). However, some thrips species have populations that are unisexual (all female) or are either unisexual or bixsexual (Ananthakrishnan, 1990).

**Biological control of thrips pests**

Thrips are highly mobile, opportunistic insects adapted to utilize intermittent resources such as those found in agroecosystems (Lewis, 1997; Mound, 1997; Morse and Hoddle, 2006). When a habitat is found, thrips feeding and reproduction rates increase dramatically and populations can reach pest status in a short period of time (Kirk, 1997). Because pest thrips occur throughout Thysanoptera, Mound (1997) speculated that the biological characteristics predisposing a species to becoming a pest are widespread within the group. Interestingly, < 1% of the described thrips species induce enough economic damage in various cropping systems to be considered pests (Morse and Hoddle, 2006). Nonetheless, this damage is estimated to cost growers billions of dollars in control costs and productivity losses (Lewis, 1997; Ullman et al., 1997). Damage to crops may be the result of direct feeding on plants as well as ovipositioning in or on plant tissue, or indirectly through the introduction of bacterial, fungal, and viral pathogens (Kirk, 1997). Visible feeding damage includes silvering or bronzing of the leaves, scarring and deformation of fruit and flowers, rind blemish, premature flower loss, reduced seed production, soiling of leaves and fruit with fecal droplets, and a reduction of pollen and fruit (Ananthakrishnan, 1984; Childers and Achor, 1995). Oviposition by females produces blotches or halos on the fruits of plants, resulting in aesthetic injury that can significantly affect the yield.
and value of a particular crop. Although direct damage can be severe for polyphagous species, most thrips are considered pests because of the indirect damage they cause to agroecosystems by introducing plant pathogens (Ullman et al., 1997).

Thysanopterans exhibit exponential population growth under optimal conditions, and many biologists concluded density dependent factors such as natural enemies were not important for controlling thrips because they were considered unable to keep up with rapid population growth (Davidson and Andrewartha, 1948a, b; Andrewartha and Birch, 1954; Mound and Teulon, 1995; Butt and Brownbridge, 1997; Loomans et al., 1997; Parella and Lewis, 1997; Parker and Skinner, 1997). However, extensive review by thrips ecologists confirmed that density-dependent parameters, particularly natural enemies, are important regulators of pest thrips populations (e.g., Nicholson, 1958; Smith, 1961; Varley et al., 1973; Stoltz and Stern, 1978; Nagai, 1990; Mound, 1997; Sabelis and Van Rijn, 1997; Funderburk et al., 2000; Funderburk, 2002; Ramachandran et al., 2001; Hansen et al., 2003; Reitz et al., 2003, 2006; Baez et al., 2004).

Arthropod predators of Thysanoptera include Neuroptera, Diptera, Hymenoptera, Coleoptera, Dictyoptera, Orthoptera, Hemiptera, Thysanoptera, and Acari (Sabelis and Van Rijn, 1997). The most common predators of Frankliniella spp. are species of Orius (Hemiptera: Anthocoridae) and Amblyseius (Acari: Phytoseiidae) (Loomans et al., 1995; Riudavets, 1995). Controlling thrips populations with these natural enemies is only partially successful because day length and body size limits their performance and both are vulnerable to insecticides (Butt and Brownbridge, 1997; Sabelis and Van Rijn, 1997). Entomogenous fungi within the Hyphomycetes (Verticillium, Metarhizium, Paecilomyces, and Hirsutella) and Zygomyces (Entomophthorales) are important yet often overlooked thrips pathogens (Parker et al., 1996;
Butt and Brownbridge, 1997). Unlike arthropod biological control agents, fungi are not limited by day length, are active over a range environmental conditions and climates, do not need to be ingested to be pathogenic, and are easily mass-produced (Parker et al., 1996; Butt and Brownbridge, 1997). The biology of fungal entomopathogens, and their potential to regulate thrips populations, is reviewed in further detail below (see *Entomophthorales*).

Some researchers have evaluated the potential of using entomopathogenic nematodes, particularly Heterorhabditidae and Steinernematidae (Rhabditida), as biological control agents because of their high level of virulence against soil-inhabiting stages of thrips (e.g., Chyzik et al., 1996; Ebssa et al., 2001; Buitenhuis and Shipp, 2005). However, high concentrations of infective juveniles are required to obtain high mortality, and although the nematodes are easily mass-produced, the numerous inundative spray applications required for thrips control are not economically feasible (Chyzik et al., 1996; Ebssa et al., 2001). Other parasitic nematodes, the entomogenous Tylenchida (=*Allantonematidae*), are naturally occurring parasites of thrips. Unlike the entomopathogenic nematodes that kill their host in a short period of time, these parasites have negligible effects on thrips longevity or mortality (Sims et al., 2005). Thus, these parasites are self-sustaining and little effort is needed to maintain field populations. The biology of allantonematid nematodes is reviewed in further detail below (see *Thripinema fuscum*).

*Frankliniella fusca* (Hinds) (Thysanoptera: Thripidae)

The first specimen of *Frankliniella fusca* Hinds was collected in Massachusetts and described as *Euthrips fuscus* by Hinds in 1902. In 1912, Karny placed *E. fuscus* in *Frankliniella*. Watson (1922) was the first to identify thrips species in Florida peanut and included *F. fusca* in his research bulletin. Currently, *F. fusca* is distributed throughout Canada, Mexico, and the eastern United States (Sakimura, 1963; Stannard, 1968; Chiasson, 1986; Johansen, 2002).

*Frankliniella fusca* can be differentiated from other species in the genus by having short or
wanting postocular bristles, the third antennal segment being yellowish and the fourth and fifth light grayish brown, the body generally dark brown to lighter brown, long, stout spines on the legs, and a lack of comb on tergite VII (Watson, 1923). There are several adult taxonomic keys available for distinguishing *F. fusca* from other *Frankliniella* species (*e.g.*, Childers and Beshear, 1992; Oetting et al., 1993; Mound and Kibby, 1998; Moritz et al., 2001, 2004); however few larval keys exist because young instars possess few defining character states. Larval identification alternatives include rearing immature thrips to adulthood and molecular analyses (Brunner et al., 2002; Paini et al., 2007).

Numerous studies have been conducted on the biology of *F. fusca* (Watts, 1934; Newsom et al., 1953; Sakimura, 1963; Stannard, 1968; Lowry et al., 1992; Puche and Funderburk, 1992; Sims, 2003; Sims et al., 2005; Sims et al., 2009). Unlike other flower thrips species, *F. fusca* are relatively sessile and easy to manipulate for laboratory experiments. The postembryonic development of *F. fusca* includes a bean-shaped egg embedded within plant tissue, two larval instars, two non-feeding inactive pupal stages termed the pre-pupa and pupa, and an adult stage that develops either macropterous or brachypterous wing pads (Figure 1-1). *Frankliniella fusca* is unique in that it is only one of a few species within Thripidae that exhibit both macropterous (wings present) and brachypterous (wings reduced to pads that range in size) wing morphs (Mound, 1996). Stannard (1968) noted minor variations in structure and color between the two wing morphs of male and female *F. fusca*; macropterous individuals are slightly larger and darker than their brachypterous counterparts. *Frankliniella fusca* are also sexually dimorphic and the larger, brown females can be easily differentiated from the smaller, yellow male adults (Figure 1-1).
The survival, development, longevity and reproduction of *F. fusca* on peanut at various temperatures (Lowry et al., 1992), as well as the intrinsic rate of increase at different population densities, have been well documented (Lowry et al., 1992; Puche and Funderburk, 1992; Sims, 2003; Sims et al., 2005). The developmental time of *F. fusca* is typical for poikilothermic organisms; a developmental cycle (egg to egg) can be completed in 12 to 24 days under optimal (35°C) and suboptimal temperatures (20°C), respectively (Lowry et al., 1992, Sims, 2003; Sims et al., 2005). Egg development requires nearly half of the total developmental time with larval emergence occurring seven days after ovipositioning between 20-30°C (Lowry et al., 1992). Overcrowded conditions reduce the survivorship of larval thrips as a result of intraspecific competition for resources (Paini et al., 2008). Adult females have a pre-vitellogenic period of one day, after which they lay an average of two eggs per day (Heming, 1970; Lowry et al., 1992; Sims, 2003; Sims et al., 2005). The longest average female longevity reported for *F. fusca* is 13 days, which can be attributed to the addition of pollen to the female’s diet (Sims et al., 2005). Male and female *F. fusca* develop at the same rate, however females live on average four days longer (Sims, 2003). *Frankliniella fusca* generally maintains a 3:1 (female: male) sex ratio, however because they exhibit arrhenotokous parthenogenesis, populations are able to survive in the absence of haploid males (Sims et al., 2005). Because of this attribute, genders are easily manipulated in laboratory populations with unmated and mated females always producing males and females, respectively.

*Frankliniella fusca* is the most common thrips species found inhabiting and reproducing in groundnut and can comprise over 90% of the total thrips population in a legume field (Funderburk et al., 1998; Herbert et al., 2007). In Florida, populations of *F. fusca* are able to reproduce year round on groundnut and can produce as many as 15 generations per year.
Larvae and adults inhabit the terminal buds and flowers, respectively, and the non-feeding pre-pupae and pupae tend to drop to the soil just below the plant. Larvae and adults feed in flowers and on the upper surface of plant leaves, causing a silvery scarring on the leaf surface and an upward curling of injured leaves (Newsom et al., 1953). Direct feeding injury to young groundnut by *F. fusca* does not result in significant yield loss unless other stressors such as post-emergence herbicide injury are present (Smith and Sams, 1977; Tappan and Gorbet, 1979; Lynch et al., 1984; Funderburk et al., 1998). Although *F. fusca* feeding damage can cause asthetic injury to plants, most crop losses are attributed to their vectoring capabilities. *Frankliniella fusca* adults colonize seedlings immediately after planting when the young plants are most susceptible to viral infection (Todd et al., 1996). Populations peak about 10 to 20 days after the planting date, and peak injury levels occur approximately 23 to 35 days after planting when peanuts start flowering (Tappan and Gorbet, 1979; Todd et al., 1996). Thrips populations begin to decrease in May and eventually reach levels near extinction in mid-summer (Tappan and Gorbet, 1979; Todd et al., 1996; Funderburk et al., 2002). Previous speculation for the population declines included physiological factors (e.g., diapauses) and natural enemies; the recent discovery of the sterilizing insect parasitic nematode, *Thripinema fuscum* Tipping & Nguyen, is now known to be one of the sources for the observed population decline (Funderburk et al., 2002).

**Entomoparasites Associated with *Frankliniella fusca***

As with all insects, Thysanopterans have associations with a suite of prokaryotic, eukaryotic, and viral microorganisms. These symbionts can be temporary or permanent residents that establish a commensalistic, mutualistic, or parasitic relationship with their thrips host. This section covers the symbioses of thrips entomoparasites and the interactions they have with their host. In this dissertation, the term “entomoparasites” refers to the viral (TSWV), bacterial
(Pantoea ananatis and Wolbachia), fungal (Entomophthorales spp.), and nematode (Thripinema fuscum) agents infecting F. fusca.

**Tomato spotted wilt virus (Bunyaviridae: Tospovirus)**

The Bunyaviridae comprises the largest family (>350 spp.) of arthropod-transmitted viruses and includes many important human pathogens such as Rift Valley fever (Phlebovirus), Crimean-Congo hemorrhagic fever (Nairovirus), California encephalitis virus (Hantavirus), and La Crosse virus (Orthobunyavirus) (Elliott, 1997). Tospovirus is the sole plant-infecting virus in the Bunyaviridae; the other genera (Bunyavirus, Phlebovirus, Hantavirus, Nairovirus, and Tenuiviruses) are vectored by hematophagous arthropods (mainly mosquitoes, ticks, and sand flies) or rodents and infect humans and animals (Sherwood et al., 2001). There is a high degree of vector specificity for Tospoviruses; all species are transmitted in a semi-persistent propagative manner exclusively by thrips in the Thripidae (Sakimura, 1963; Ullman et al., 1992; Nagata et al., 2002). Currently, the Tospovirus genus is composed of 19 species (Pappu et al., 2009).

Species classification within the Tospovirus are primarily determined by the nucleocapsid (N) gene sequence although other factors such as host range, serological differences, and genome structure and organization have been used (Elliot, 1990; de Avila et al., 1990, 1993). Of the estimated 5,500 thrips species, 10 are known vectors of Tospoviruses (Pittman, 1927; Sakimura, 1963; Mound, 1996; Ullman et al., 1997; Whitfield et al., 2005). Most thrips Tospovirus vectors are restricted to Thrips and Frankliniella genera within Thripinae, and this vectoring ability is thought to have evolved independently in the two lineages (Mound, 1996). Tomato spotted wilt virus (TSWV), the type member of Tospovirus, is economically the most important species within the genus. This pathogen is considered to be among the 10 most detrimental plant viruses worldwide and annual crop loss from TSWV has been estimated to exceed $1 billion (Goldbach and Peters, 1994; Prins and Goldbach, 1998). Tomato spotted wilt virus was first discovered in
1915 infecting tomatoes in Australia, found to be transmitted by thrips in 1927, and to be of viral origin in 1930 (Brittlebank, 1919; Pittman, 1927; Samuel et al., 1930). Currently, there are seven substantiated thrips vectors of TSWV – *F. fusca* (Sakimura, 1963), *F. intonsa* (Trybom) (Wijkamp et al., 1995), *F. occidentalis* (Gardner et al., 1935), the dark form of *F. schultzei* (Trybom) (Sakimura, 1969), *Thrips palmi* Karny (Fujisawa et al., 1988), *T. setosus* Moulton (Fujisawa et al., 1988), *T. tabaci* Lindeman (Pittman, 1927), and *F. bispinosa* (Morgan) (Avila, 2006). Other reported thrips-TSWV associations have been invalidated by Mound (1996), who cited a misidentification of thrips species by the authors. There are undoubtedly additional vectors of TSWV within the Thripidae, as well as new vectors that will evolve as the Tospovirus-vector association changes over time. *Thrips tabaci*, for example, was at one time a primary vector but has since lost the ability to vector new isolates (Sakimura 1962, 1963; Mau et al., 1990; Wijkamp et al. 1995). These new isolates are formed when a host plant is coinoculated with different isolates that exchange genetic information through a reassortment of genome segments (Qui et al., 1999).

Tospoviruses are classified as single stranded, negative-sense tripartite RNA viruses (Class V of the Baltimore Classification of viruses). The spherical to pleomorphic virions measure 70-80 to 90-120 nm in diameter and the outer shell of the virion consists of a host derived double-layered membrane or envelope (5 nm thick) anchored with two viral glycoproteins (5-10 nm long) (Milne, 1970; Whitfield et al., 2005). The envelope encloses three non-covalently closed, circular ribonucleoproteins that are composed of the ssRNA segment, the nucleoprotein and multiple copies of the viral RNA-dependent RNA polymerase (Fauquet et al., 2005; Whitfield et al., 2005). The three linear single stranded RNA segments, S (2.9 kb), M (4.8 kb), and L (8.9 kb), code for four structural proteins and two nonstructural proteins from five open reading
frames. Collaborations between de Haan et al. (1990, 1991) and Kormelink et al. (1992) resulted in the fully sequenced genome of TSWV and provided a greater understanding of the viral organization, replication, and function as herein described. The ambisense S segment codes for a 52.4 kDa nonstructural protein (NSs) in the viral sense and a 29 kDa nucleocapsid protein (N) in the viral complementary sense (De Haan et al., 1990). The NSs functions as a silencing suppressor, and because this protein is not present in the mature virion, it is often used as a marker for viral replication in serological and molecular studies (de Haan et al., 1990; Kormelink et al., 1991; Kikkert et al., 1997; Takeda et al., 2002). The N protein is highly conserved among all TSWV isolates and is the primary gene used to for identification in molecular studies. The ambisense M segment codes for a 33.6 kDa nonstructural protein (NSm) in the viral sense and a 127.4 kDa precursor of the two glycoproteins (GP1 and GP2) in the viral complementary sense (Kormelink et al., 1992). The NSm protein is involved in cell-to-cell movement through plasmodesmata and acts as an avirulence determinant (Kormelink et al., 1994; Storms et al., 1995, 1998; Margaria et al., 2007). The glycoproteins coordinate the TSWV entry process into thrips tissues and are required for transmissibility by thrips (Bandla et al., 1998; Medeiros et al., 2000; Sin et al., 2005; Ullman et al., 2005). The negative-sense L segment codes for a 331.5 kDa RNA-dependent RNA polymerase (RdRp) (de Haan et al., 1991; Adkins et al., 1995).

The TSWV pathway in the thrips vector is still unresolved, but the viral mode of infection has been shown to be systemic and time-dependent (Moritz et al., 2004; Whitfield et al., 2005). The acquisition access period (AAP) and the inoculation access period (IAP) are two important determinants for viral transmission by the thrips vector (Wijkamp et al., 1995, 1996; van de Wetering et al., 1996). Acquisition of TSWV occurs when first and early second instars ingest virus particles from infected plant tissue (van de Wetering et al., 1996). The minimum AAP
reported for successful transmission is five minutes, although transmission rates are highest after an AAP of approximately 24 hours (Wijkamp et al., 1996). Young first instars are most susceptible to infection and the acquisition efficiency decreases with increased larval age (van de Wetering et al., 1996). When young larvae feed on infected plant tissue, the ingested virions migrate from esophagus to the midgut where glycoprotein one (GP1) located on the viral envelope bind to columnar epithelial cells through a 50 kDa cellular receptor located on the plasmalemma (Bandla et al., 1998; Medeiros et al., 2000). The virus fuses to the cell, possibly through pH-dependent receptor-mediated endocytosis, and releases its replicative contents into the cell (Bandla et al., 1998; Whitfield et al., 2005). Viral mRNA is transcribed using the host machinery and the virion-associated RdRps, assembled in the Golgi complex, and disseminated between cells via the exocytic pathway (Kikkert et al., 1999; Whitfield et al., 2005). The virus spreads throughout the midgut and foregut, moving from the midgut epithelia to the surrounding visceral muscle cells (Kritzman et al., 2002). At this point, the route of infection from the midgut cells to the salivary glands is speculative. The general consensus among thrips biologists is that virus movement is ontogenic-dependent, occurring in first instars when displacement of the salivary glands by the cibarial muscles causes a temporary fusion of the midgut to the visceral muscle cells surrounding the salivary glands (Moritz et al., 2004). However, other viruses (e.g., Rhabdoviruses) are neurotropic in their insect hosts and the possibility that TSWV utilizes neurons and trachea to reach these glands cannot be ruled out (Ammar and Hogenhout, 2008; Ammar et al., 2009). This dissemination may occur at the midgut/muscle border because the gut epithelia are closely associated with the insect tracheal system (Ammar et al., 2009). It was reported that the salivary organs formed a channel-like structure to the midgut and the virus utilized these ligaments as a means for accessing the salivary tissues, however in-depth
ultrastructural examination showed there is actually no mergence between the tubular salivary glands and the midgut (Ullman et al., 1989; Del Bene et al., 1999). Movement of TSWV through the hemolymph has also been ruled out through viral injection studies (Nagata and Peters, 2001). Receptors are widely distributed among different cell types and the binding of glycoprotein 2 (GP2) to 94 kDa thrips protein is likely involved in circulation of the virus within thrips tissues (Kikkert et al., 1998; Ammar et al., 2008). Studies conducted by Sin et al. (2005) have shown that both viral membrane GP1 and GP2 are required for thrips transmissibility. The ovoid salivary glands are the primary site of replication for the virus and they must be heavily infected for successful transmission (Wijkamp et al., 1993; Nagata et al., 1999; Nagata and Peters, 2001). Transmission occurs when mature virus particles are released with saliva into a healthy plant cell during feeding by second stage larvae and adults (Wijkamp et al., 1993). Thrips TSWV transmission rates are high even after short inoculation periods which can have significant implications for disease spread in field conditions. Healthy adults do not become viruliferous after feeding on infected plant tissue even after long periods of exposure (Sakimura, 1963). Moritz (1997) suggested the inaccessibility of late larval and adult stages to acquire and transmit the virus is due to the extending and thickening of the peritrophic membrane with development, causing it to be less permeable to viruses. Sherwood et al. (2001) and van de Wetering et al. (1999) suggest differences in acquisition capabilities between larvae and adults reflect differences in feeding behavior. However, Ullman et al. (1992) found that both larval and adult thrips sufficiently ingest TSWV, and instead concluded that adult thrips are unable to acquire the virus due to the presence of a midgut barrier. They observed that when larvae feed on infected plant tissue, virions enter the midgut epithelial cells by fusion at the apical membrane of the midgut epithelial brush border (Ullman et al., 1995). However, when adults feed the
virions are degraded or altered in the midgut and/or epithelial cells so dissemination to other tissues cannot occur. Additional support for the barrier hypothesis comes from work conducted by Nagata et al. (1997) who were able to develop tissue cell cultures supporting TSWV multiplication from the non-vectoring *T. tabaci*. To conclude, the virus must penetrate through 7 internal barriers for successful acquisition and transmission to occur: (1) peritrophic envelope/laminae, (2) apical membrane of brush border in midgut lumen, (3) basement membrane of midgut columnar epithelial cells, (4) midgut basal lamina, (5) through the surrounding muscle cells, (6) basal lamina of salivary glands, and (7) plasma membrane of salivary gland secretory cells (Ullman et al., 1992; Ammar et al. 2009). Disrupting viral entry, movement, development, replication, or escape from any of these barriers can affect the vectoring capability of an individual (Nagata et al., 1999; Ananthakrishnan and Annadurai, 2007).

The vector competence of thrips is dependent upon other intrinsic factors including the rate of viral multiplication in the insect, feeding behavioral differences, gender, genetic elements of both the virus and vector, insect immunity, and ontogeny (Hardy et al., 1983; Nagata et al., 2002; Arthurs and Heinz, 2003; Medeiros et al., 2004; Moritz et al., 2004; Cabrera-La Rosa and Kennedy, 2007; Rotenberg et al., 2009; Sims et al., 2009). *Tomato spotted wilt virus* titer in host thrips has been shown to be a quantitative determinant of vector competence, and the existence of a dose-dependent infection threshold has been documented in other insect-RNA virus associations such as mosquitoes and arboviruses (Hardy, 1983; Rotenberg et al., 2009). Differences in feeding behavior, particularly those between genders, often dictate the amount of virus secreted to infected plant cells. Sakuri et al. (1998) found that males have a higher transmission rate than females and suggested that because females feed intensively, they destroy
cells to the extent that virus replication is not supported in the plant cell. Males, on the other hand, puncture the cells and leave them suitable for virus replication and consequent infection of the plant (van de Wetering et al., 1999). Differences in transmission efficiency between genders was further supported by work conducted by Rotenberg et al. (2009), who found males transmitted TSWV at a greater rate than females but harbored less TSWV-N RNA. Host genetic elements may also explain vector competence and has been well documented in other systems such as mosquito vectors and the pathogens they transmit (Gray and Banerjee 1999; Beerntsen et al., 2000; Sim et al., 2009). Cabrera-La Rosa and Kennedy (2007) suggest vector competence to be under genetic control and inherited as a recessive trait, however sexually transmitted factors other than genes may be influencing thrips vectoring efficiency (e.g., Wolbachia). Medeiros et al. (2004) found that TSWV infection activates the insect innate immune system and the elicited defense responses are effective in resisting infection. The developmental stage in which the virus is ingested is another critical step in determining viral competence. Extrinsic factors, such as density and environmental conditions, also affect whether an insect vector will acquire and transmit viruses (Hardy et al., 1993). For example, stable environmental conditions typically produce brachypterous thrips; these individuals probably do not transmit TSWV as efficiently as macropterous thrips because of their limited dispersal and contact capabilities (Wells et al., 2002a).

Tospovirus epidemics can occur only when the thrips vector, tospovirus, and host plant coincide in a suitable environment (a.k.a. “disease triangle”) (Ullman, 1996). *Tomato spotted wilt virus* is currently known to infect over 900 monocotyledonous and dicotyledonous plants and both virus and host plants are distributed worldwide (Sherwood et al., 2001; Culbreath et al., 2003; Campbell et al., 2009; Pappu et al., 2009). The large, overlapping host ranges of the virus
and vector increases the probability that a vector will contact an infected plant and makes this pathosystem extremely difficult to control (Mound, 1996; Pappu et al., 2009). *Tomato spotted wilt virus* was first reported on Florida groundnut in June of 1986, although it may have occurred there as early as 1974 (Kucharek et al., 1990). *Frankliniella fusca* is the key vector of TSWV in Florida groundnut and causes extensive economic damage (Funderburk et al. 2002). *Tomato spotted wilt virus* cause systemic infection in their plant hosts and symptoms include circular light green and yellow ring spots on the foliage, yellowing of the leaves, tan spots or blotches, streaking or mottling of the quadrifolates, deterioration of the root system, shrinking of the leaves, and an overall reduction in size of the plant (Figure 1-2) (Culbreath et al., 1993; Padgett et al., 1995). *Tomato spotted wilt virus* epidemics spread through groundnut fields in two main ways. Primary spread occurs when viruliferous thrips disperse to newly planted crops from surrounding vegetation and transmit the virus to healthy plants. The control of primary spread of TSWV by viruliferous thrips is considered to be a key component in disease management; most losses in groundnut fields result from an inability to control primary spread (Camann et al., 1995; Puche et al., 1995; Gitaitis et al., 1998; Culbreath et al., 2003). Secondary spread occurs when larvae developing on infected plants within a crop acquire the virus and transmit it to other uninfected plants in the field after they have matured to adulthood. The control of TSWV secondary spread relies heavily on the application of broad-spectrum insecticides. However, insecticides alone do not prevent TSWV primary spread because adult thrips feed and transmit before insecticidal activity can kill the vector (Todd et al., 1996; Momol et al., 2004). Application of insecticides for thrips control also has been shown to have negative impacts on beneficial insects and can lead to thrips resistance (Newsom et al., 1953; Bielza et al., 2007, 2008). No single control measure has been effective in significantly reducing thrips transmitted
disease spread and many growers have implemented integrated control management systems that combine chemical, biological, and cultural practices (Sherwood et al., 2001; Culbreath et al., 2003). Cultivar selection, planting date, field location, plant population, row pattern, tillage, weed control, UV-reflective mulch, and insecticides are all strategies used to minimize losses of crop plants (Stavisky et al., 2002a; Culbreath et al., 2003; Reitz et al., 2003; Momol et al., 2004; Riley and Pappu, 2004). Additional control measures, especially those utilizing natural enemies, are needed for reducing pest thrips in agroecosystems.

Annual cropping systems fluctuate in their availability and suitability to insect pests (Kennedy and Storer, 2000). In winter months, *F. fusca* populations are highly brachypterous and commonly overwinter around harvested groundnut fields where volunteer groundnut and annual weed species take over (Newsom et al., 1953; Chamberlin et al., 1993). The ability of *F. fusca* to feed on a wide variety of host plants is a critical factor that promotes thrips dispersal and allows for bi-directional movement between cultivated (crop) and non-cultivated (weeds) host plants over time and space. Many alternate host plants serve as reservoirs for both TSWV and the *F. fusca* vector, allowing for virus acquisition and transmission between generations of thrips and crops (Chamberlin et al., 1992, 1993). Many publications have discussed the distribution and abundance of *F. fusca* in these cultivated and uncultivated host plants (Chamberlin et al., 1992; Chellemi et al., 1994; Buntin and Beshear, 1995; Toapanta et al., 1996; Groves et al., 2001; Groves et al., 2002; Kahn et al., 2005; Paini et al., 2007; Northfield et al., 2008).

Transmission of TSWV is limited in brachypterous adults because of their inability to disperse long distances. Therefore, unless thrips diapause or emigrate from other regions, these winter plants and thrips are the primary means for TSWV to move across seasonal crop harvests. Dispersal from a host plant is dependent upon food, mates, oviposition sites, natural enemies,
and/or density. A major factor influencing dispersal from overwintering host plants is weather (Lewis, 1964; Puche and Funderburk, 1995). High temperatures promote dispersal and movement within a groundnut field whereas cool temperatures and/or rainfall induces stagnant populations (Harding, 1961). Increased photoperiod and temperatures associated with spring conditions encourages the development of winged individuals, and the highest dispersal rates frequently correspond with the highest level of macropterous individuals (Newsom et al., 1953; Hood, 1940; Koppa, 1970; Kamm, 1972; Chamberlin et al., 1992; Mound, 2005). This dispersal usually coincides with early stages of crop growth when the plants are most prone to damage (Northfield et al., 2008; Pappu et al., 2009). The pattern of dispersal into a groundnut field appears to be associated with the prevailing wind rather than the location of the overwintering crop (Garcia and Brandenburg, 1995). However, one can discern the movement for viruliferous thrips by analyzing the spatial distribution of infected plants in the field (Puche et al., 1995). Vectors arriving from distant sources produce a random distribution of diseased plants throughout the field whereas vectors from within the field tend to produce a clumped or aggregated pattern of diseased plants. Vectors coming from weed hosts bordering the field tend to generate a gradient of infected plants from the edge of the field (Puche et al., 1995).

*Pantoea ananatis* (Serrano 1928) Mergaert et al. 1993

Many phytophagous insects have facultative or obligate mutualistic associations with bacteria. For thrips, this relationship appears to be specific to the plant pathogenic *Pantoea* spp. (McKenzie et al., 1993; de Vries et al., 2001a,b, 2004, 2008; Wells et al., 2002b; Gitaitis et al., 2003; Chanbusarakum and Ullman, 2008, 2009). *Pantoea ananatis* has been isolated from the guts of other insects such as plant hoppers, pyramids, and flea hoppers (Takahashi et al., 1995; Watanabe et al., 1996; Bell et al. 2007). *Pantoea ananatis*, first described as *Erwinia ananas* (syn. *E. ananatis, E. herbicola, E. uredovora*), was discovered causing fruitlet brown-rot of
pineapple in the Philippines. Serrano (1928) placed this pathogen into the herbicola group of the *Erwinia* genus, a group proposed by Winslow et al. (1920) for “all plant-associated, gram-negative, non-spore forming, peritrichous, fermentative, rod-shaped bacteria”. The species was moved to the genus *Pantoea* in 1989, and renamed *P. ananatis* and *P. agglomerans* (Beijerinck 1888) in 1997 (Gavini et al., 1989; Mergaert et al., 1993; Truper and De’Clari, 1997).

Ullman et al. (1989) first detected gram-negative bacteria in the gut of *F. occidentalis*, and the bacteria were later isolated and characterized by de Vries et al. (1995, 2001a). These microbes reside in the hindgut and Malpighian tubules of all thrips life stages (Ullman et al., Dallai et al., 1991, 1989; de Vries et al. 2001a). Transmission of the gut bacteria occurs horizontally on the food source from feces or saliva, or vertically through contamination of the egg shell (de Vries et al., 2001b). Currently, associations with *Pantoea* spp. gut bacteria are known for *F. occidentalis*, *F. fusca*, and *T. tabaci* (Ullman et al., 1989; de Vries et al., 2001a, b; Wells et al., 2002; de Vries et al., 2008; Chanbusarakum and Ullman, 2008, 2009). Interestingly, *F. occidentalis* and *F. fusca* are rarely infected by any other gut bacteria (de Vries et al., 2001a, b; Wells et al., 2002; Chanbusarakum and Ullman, 2009). These bacterial complexes are stable over time and space, suggesting the bacterial association is both symbiotic and widespread among *Thrips* and *Frankliniella* species (de Vries et al., 2008; Chanbusarakum and Ullman, 2008, 2009).

In 2002, isolates of *P. ananatis* recovered from laboratory and field collected *F. fusca* were shown to be pathogenic to onion (Wells et al., 2002). One year later, Gitaitis et al. (2003) reported that *F. fusca* is an important vector of the plant pathogenic *P. ananatis*. Similar to TSWV, *P. ananatis* is an emerging plant pathogen that infects both monocotyledonous and dicotyledonous plants and causes disease symptoms in economically important agricultural crops.
These bacteria have a quorum sensing system, a cell-to-cell communication by bacteria that determines local cell density, and the biosynthesis of exopolysaccharides as a quorum-sensing signal molecule induces the onion rot disease (Morohoshi et al., 2007). This pathogen is broadly adapted to habitats and can function as an epiphyte, endophyte, pathogen, symbiont, and/or saprophyte (Coutinho and Venter, 2009). *Pantoea ananatis* functions as both a saprophyte inhabiting the gut microfauna of *F. fusca* and as a pathogen causing leaf blight, necrotic lesions, stalk rot, and bulb decay in onion (Wells et al., 2002; Walcott et al., 2002; Gitaitis et al., 2003). The severity of *P. ananatis* is highly dependent on environmental conditions (*e.g.*, temperature, humidity, and precipitation) and causes 100% crop loss under favorable conditions (Gitaitis and Gay, 1997; Walcott et al., 2002). Once established in a field (*e.g.*, by seed inoculums), *P. ananatis* likely spreads between crops from the movement of thrips vectors. For example, both Vidalia onion and groundnut are hosts for *P. ananatis* and *F. fusca* (Watson, 1922; Sether and DeAngelis, 1992; Gitaitis and Gay, 1997; Wells et al., 2002; Mullis et al., 2004). Current control measures for *P. ananatis* include developing resistant cultivars, using mulch and irrigation, and avoidance and eradication of initial inoculums (Gitaitis et al., 2004; Coutinho and Venter, 2009).

**Wolbachia Hertig (Rickettsiales: Rickettsiaceae)**

The intracellular bacterium *Wolbachia* was first discovered in 1924 infecting the reproductive structures of *Culex pipiens* L. mosquitoes and are now one of the most abundant and widespread bacterial symbionts known (Hertig and Wolbach, 1924; Werren, 1997). In addition to insects, *Wolbachia* has been found in crustaceans, mites, spiders, scorpions, filarial nematodes, and a plant nematode (McLaren et al., 1975; Breeuwer and Jacobs, 1996; Cordaux et al., 2001; Rowley et al., 2004; Baldo et al., 2007; Haegeman et al., 2009). Estimates suggest *Wolbachia* infect 20 to 66% of all arthropods species; however this estimate is higher when...
testing for *Wolbachia* using modified PCR detection methods (Jeyaprakash and Hoy, 2000; Hilgenboecker et al., 2008). A variety of protocols have been developed for *Wolbachia* detection in arthropods; the most reliable and accurate protocols include histological examination, phenotypic identification, and multi locus sequence typing (MLST) of five *Wolbachia* specific genes (Hertig and Wolbach, 1924; Baldo et al., 2006) (see Chapter 2).

*Wolbachia* belong to the alpha subdivision of the Proteobacteria, and are currently divided into nine clades or ‘supergroups’ based on nucleotide sequence data (Weisburg et al., 1989; Lo et al., 2007; Ross et al., 2009). All *Wolbachia* strains within the supergroups represent the single species *W. pipientis* (Lo et al., 2007). These bacteria are found in cytoplasm of the host reproductive cells and are predominantly transmitted vertically from the mother to offspring (Wade and Stevens, 1985). By targeting their host reproductive system, *Wolbachia* employ a variety of strategies to enhance their transmission such as cytoplasmic incompatibility, feminization, parthenogenesis, and male killing (Werren, 1997). These effects bias population sex ratios and may even cause speciation (Werren, 1997). In addition to manipulating host reproduction, *Wolbachia* has been shown to induce the up-regulation of host immune genes (Brattig et al., 2004; Kambris et al., 2009). This up-regulation, in turn, inhibits the establishment of other microorganisms such as nematodes, bacteria, and viral infection (Lowenberger et al., 1996; Kambris et al., 2009; Teixeria et al., 2008) (see Chapter 5).

Although vertical transmission from mother to offspring is the primary route for *Wolbachia* infection, there is ample evidence in the literature of horizontal transmission between taxa. This exchange may occur through intimate associations between hosts and their parasitoids (see Werren et al., 1995; Heath et al., 1999; Vavre et al., 1999), prey (see Johanowicz and Hoy 1996), host plants (see Sintupachee et al., 2006), or parasites (see Noda et al., 2001). Lateral
gene transfer is also widespread, and *Wolbachia* inserts have been found in *Drosophila*, wasps, nematodes, and other bacterial symbionts (Werren et al. 1995; Dunning Hotopp et al., 2007; Darby et al., 2010). Nearly the entire *Wolbachia* genome has been transferred to its *Drosophila* host (Dunning Hotopp et al., 2007). Gene transfer through the bacteriophage WO has also been demonstrated (Bordenstein and Wernegreen, 2004). Additionally, there is significant inter- and intra-specific variation in *Wolbachia* infection; individuals within a populations can range from not being infected to being infected with multiple isolates (Boyle et al., 1993; Hilgenboecker et al., 2008; Kawasaki et al. 2009). Individuals infected with multiple *Wolbachia* strains are thought to have risen by homologous recombination (Kondo et al., 2002; Reuter and Keller, 2003; Dunning-Hotopp et al., 2007). The ability of *Wolbachia* to undergo extensive gene recombination between strains suggests it may be used as a biological control against insect vector pest populations by serving as a vehicle for introducing anti-viral genes into insect tissues that could interfere with pathogen replication or transmission (Beard et al., 1993; Werren and Bartos, 2001).

In most arthropods, these reproductive parasites are considered facultative pathogens as cured individuals are physiologically unaltered (Werren, 1997; Duron et al., 2008). However, mutualistic associations with arthropods do exist. For example, *Wolbachia* are thought to function as nutritional mutualists by providing a fecundity benefit to dietary-stressed *Drosophila melanogaster* Meigen (Brownlie et al., 2009). In fact, some of these mutualistic associations are obligatory. Antibiotic clearing of *Wolbachia* in *Asobara tabida* Nees (Hymenoptera: Braconidae) inhibits oogenesis and it is thought *Wolbachia* interacts directly with the Sex-lethal (Sxl) gene required for oogenesis (Dedelne et al., 2001; Starr and Cline, 2002). Recently, antibiotic clearing of *Wolbachia* in the collembolan *Folsomia candida* was shown to induce
sterility in all individuals despite the production of eggs (Pike and Kingcomobe, 2009). Filarial nematodes also have an obligatory mutualistic association with *Wolbachia*, with antibiotic treatment resulting in negative effects to the worm’s reproduction and development (Hoerauf et al., 1999; Taylor and Hoerauf, 1999). Complete genome sequencing of *Brugia malayi* helped to delineate the mutualistic association between *Wolbachia* and its nematode host; *Wolbachia* provides nematodes with essential metabolites such as riboflavin, flavin adenine dinucleotide, and heme, while the nematode supplies amino acids required for *Wolbachia* growth (Foster et al., 2005). *Wolbachia* are not obligatory symbionts for all filarial nematodes, as demonstrated by the inability to find the bacteria in some species such as *Loa loa* (Buttner et al., 2003). To date, *Wolbachia* has only been detected in one non-filarial nematode, the banana-root nematode *Radopholus similis* (Cobb) (Tylenchida: Pratylenchidae), and has yet to be detected in Tylenchid nematodes (Bordenstein et al., 2003; Duron and Gavotte, 2007; Foster et al. 2005; Haegeman et al., 2009).

*Wolbachia* has been reported in both arrhenotokous [e.g., *Echinothrips americanus* Morgan, *Gynaikothrips ficorum* (Marchal), *Suocerathrips linguis* Mound and Marullo] and thelytokous [e.g., *Franklinothrips vespiformis* (Crawford DL), *Hercinothrips femoralis* (Reuter), *Heliostrips hemorrhoidalis* (Bouche), *Parthenothrips dracaenae* (Heeger)] thrips species and is hypothesized to be the causal agent for the observed parthenogenesis (Pintureau et al., 1999; Arakaki et al., 2001; Kumm and Moritz, 2008). High temperature or antibiotic treatment removed *Wolbachia* from thelytokous females and induced the production of sexually functional males (Arakaki et al., 2001; Kumm and Moritz, 2008). Unfortunately, little is known regarding the effect of *Wolbachia* on the reproductive behavior of arrhenotokous populations of thrips (see Chapter 2).
Entomophthorales spp.

Fungi isolated from, or proven pathogenic to, thrips are in the Hyphomycetes or Zygomycetes classes (Butt and Brownbridge, 1997). Despite the abundance of information on fungal pathogens of thrips (particularly *F. occidentalis*) (for a review see Butt and Brownbridge, 1997), there are few reports of fungi associated with *F. fusca* in field conditions. However, I have recovered a variety of parasitic and saprophytic fungal pathogens from *F. fusca* populations in north central Florida groundnut (Figure 1-3). The most common mycopathogen associated with *F. fusca* is *Entomophthorales* spp. (*Zygomycota: Entomophthoraceae*). *Entomophthorales* spp. are common obligate pathogens of insects, and many species have strict host specificity often restricted to a single species (Alexopoulos et al., 1996). As the name implies (“entero”=insect, “phthor”=destroyer), these pathogens are highly virulent and induce host mortality a few days after infection. These fungi can produce rapid mass epizootics in insect populations under conditions of high humidity (Bellini et al., 1992). For these reasons, fungal pathogens in this order are the focus of many biological control studies (Leathers et al., 1993).

The life cycle of *Entomophthorales* involves two forms of conidia in the *F. fusca* host (Figure 1-4; Figure 1-5). Large primary conidia are forcibly ejected from the insect cadaver, producing a halo of protoplasm around the body. Smaller secondary conidia may develop from the primary conidia if a suitable substrate is unavailable for the primary conidia. These secondary conidia, termed capilliconidia, are elongated cells produced on a long, thin conidiophore that function as an attachment organ. After the conidia attach to a suitable host, they germinate and a germ tube penetrates the insect cuticle. Once inside the insect, the mycelia fragment into hyphal bodies that rapidly multiply in the insect (Figure 1-6). Within just a few days the hyphal bodies proliferate throughout the host, eventually breaking the cuticle and killing the insect (Alexopolous et al., 1996) (Figure 1-7).
Thripinema fuscum (Tylenchida: Allantonematidae)

Nematodes have an evolutionary history of ~750 million years, and the development of the protrusible stylet in ancestral forms laid the foundation for the evolution of the parasitic nematodes (Blaxter et al., 1998; Wang et al., 1999; Siddiqi, 2001; Davis et al., 2004). Insect parasitism is thought to have arisen independently in four major groups of nematodes and it is speculated that the Tylenchida arose from ancestors that had alternations of free-living and parasitic generations on lower animals and plants (Poinar, 1975; Dorris et al., 1999; Blaxter et al., 1998; Stock). The earliest fossil evidence of insect parasitism by a Tylenchid nematode was of a Howardula species (Tylenchida: Allantonematidae) parasitic in the body cavity of a phorid fly, recovered from Baltic amber dated at approximately 40 million years (Poinar, 2003). The first record of an insect-parasitic nematode association was in 1602, and it was not for three more centuries that these microorganisms were considered for controlling insect pests (Aldrovandus, 1602; Glaser, 1931). Currently, there are 27 families of nematodes in eight orders that have associations with invertebrates that include the Tylenchida, Rhabditida, Spirurida, Strongylida, Oxyurida, Ascaridida, and Mermithida (Nguyen, 2010). All insect parasitic Tylenchida are placed in the superfamily Sphaerularioidea and the family Allantonematidae represents the basic type of Sphaerularioidea (Remillet and Laumond, 1991). Allantonematid nematodes are specialized, obligate parasites that attack their host within the most microhabitats of plant structures (Poinar, 1975; Loomans et al., 1997). They are termed ‘entomogenous’ nematodes because of their negligible effects on host longevity and mortality (Harry Kaya, personal communication).

The earliest record of a described entomogenous nematode occurring in thrips spp. was in 1895 when Uzel found a specimen infecting Thrips physopus L. in Germany (Sharga, 1932). In 1910, Jones found a nematode in California infecting Heliothrips fasciatus Pergande (Russell,
Another nematode was discovered in Russia infecting *Stenothrips graminum* Uzel in 1926 (Kolobova, 1926). Unfortunately, none of these species were named due to lack of details, descriptions, or figures (Sharga, 1932; Loomans et al., 1997). The first detailed study of the nematode *Tylenchus aptini* (Sharga) infecting *Aptinothrips rufus* (Gmelin) was reported by Sharga (1932). Lysaght (1936) later proposed the taxon should be revised to *Anguillina aptini*. The species was afterwards transferred by Wachek (1955) to the genus *Howardula*. Nickle and Wood (1964) found a parasitic nematode infecting two species of blueberry thrips, *F. vaccinii* Morgan and *Taeniothrips vaccinophilus* Hood. Although their specimens were smaller than those discovered by Sharga, they determined the parasite to be *Howardula aptini* (Sharga). In 1972, Wilson and Cooley (1972) discovered *H. aptini* infecting *F. occidentalis* in Texas. *Howardula aptini* was again found infecting a new *Megalurothrips* sp. in South India in 1982 (Reddy et al., 1982), but this species differed slightly from that of Sharga (1932) and Nickle and Wood (1964). Siddiqi (2000) then changed the genus to *Thripinema* and separated the nematodes as *Thripinema renirao*, *T. aptini*, and *T. nicklewoodii*, respectively. A new species, *T. khrustalevi*, was found parasitizing *Thrips trehernei* Prisner and *T. physopus* L. in Moscow (Chizhov et al., 1995). In 1997, a new nematode in the genus *Thripinema* was found parasitizing females of *Thrips obscuratus* (Crawford) in New Zealand (Teulon et al., 1997). Also, Tipping et al. (1998) discovered a nematode infecting an adult female *F. fusca* (Hinds) and named the new species *T. fuscum* Tipping and Nguyen. In 2000, Funderburk et al. (2002b) discovered a subspecies of *T. khrustolevi* parasitizing *F. australis* (Morgan) in Chile. To date, there are five described *Thripinema* species that parasitize thirteen species of thrips (Lim and Van Driesche, 2005). There are undoubtedly other undescribed species belonging to this little-studied genus.
Laboratory and field studies have addressed various biological aspects of the *F. fusca* and *T. fuscum* interaction (Funderburk et al., 2002; Sims, 2003; Sims et al., 2005; Funderburk and Sims, 2005; Sims et al., 2009). *Thripinema fuscum* parasitizes all stages of *F. fusca*, with adult females being the most preferred host stage and males the least preferred (Sims et al., 2005). The intrinsic capacity of increase for *T. fuscum* when parasitizing adult females and males is 0.37 and 0.34, respectively (Sims et al., 2005). More than one *Thripinema* female is capable of parasitizing a single thrips host and the *in vivo* life cycle is approximately nine days (Sims et al., 2005). Parasitism does not affect survival or longevity of female *F. fusca* (Sims et al., 2005).

Parasitism by *Thripinema* is initiated when a free-living infectious female, or “motherworm”, penetrates a thrips host with her stylet through the coxal cavities or the soft, intersegmental membranes of the thorax or abdomen (Tipping et al., 1998; Lim et al., 2001). After ingress, the motherworm undergoes a dramatic morphological transformation in the host hemocoel, in which she converts from a slender, vermiform shape to an obese form comprised of a single swollen ovary (Tipping et al., 1998) (Figure 1-8 A-B). Significantly, development of the parasitic female is synchronized with the host, as demonstrated by differences in developmental time when parasitizing different stages of thrips; parasitic females entering early or late instars do not differentiate into the reproductive (ovarian) phenotype until the thrips begin to develop their adult reproductive organs (Remillet and Laumond, 1991; Siddiqi, 2000; Sims et al., 2005). The stylet, esophagus, and alimentary system of the motherworm atrophies, and the protective cuticle is shed and replaced with a thick, microvillar layer (Subbotin et al., 1993, 1994; Subbotin and Chizhov, 1996). Limited ultrastructural studies on females of *Skarbilovinema laumondi* (Tylenchida: Iotionchiidae), a parasitic Tylenchid similar to *Thripinema*, revealed a well-developed hypodermis containing an external or ‘spongy’ layer of numerous interwoven
microvilli that are presumed to aid in the uptake nutrients required for egg production (Subbotin et al., 1993). Studies on the integument structure of other insect parasitic Tylenchid females also reveal the presence of microvilli on the hypodermal surface, although the size, shape, and arrangement of the microvilli vary (Subbotin et al., 1994; Subbotin and Chizhov, 1996). In one instance, *Howardula phyllotretae* have ampullae formed from the invagination of the hypodermal membrane that presumably, like microvilli, increase surface area for absorption of nutrients (Subbotin and Chizhov, 1996). Some Tylenchids also have an extracellular substance secreted by the hypodermis of parasitic females and juvenile nematodes that may aid in degrading host immune factors before these reach the absorptive microvilli (Subbotin et al., 1993, 1994). Parasitic females produce eggs within four to five days after ingress and typically have two to three eggs in their ovary at one time. These females are highly fecund; as many as 420 eggs have been reported in a single thrips host (Reddy et al., 1982; Loomans et al., 1997). Eggs are produced continuously until death of either the host or the parasite, although the number of eggs produced decreases substantially during the later stages of parasitism (Mason and Heinz, 2002; Sims, 2003; Sims et al., 2005). The eggs hatch in the host hemocoel, and the progeny nematodes feed on hemolymph in the thrips abdominal cavity and mature through three juvenile stages (Figure 1-8 C-G) (Tipping et al., 1998). Fully parasitized thrips contain all life stages of *Thripinema* and more than one *Thripinema* female is capable of parasitizing a host (Lim et al., 2001; Sims et al., 2005). The developed juveniles are believed to migrate from the hemocoel into the alimentary tract by using their stylet to breach tissue barriers, and, presumably, they exit via the anus or ovipositor as adults (Sharga, 1932; Lysaght, 1936; Reddy et al., 1982). Sharga (1932) reported seeing *T. aptini* juveniles in the abdominal hemocoel use their stylets to bore from the midgut or oviduct to the pyriform rectum where they apparently
remained for a period of time before they exited through the anus with the insect’s frass. Reddy et al. (1982) observed *H. aptini* exiting through *Megalurothrips* sp. ovipositor. The partially free-living females are small (0.25 to 0.29 mm long) with a hypertrophied stylet, however males have a non-functional and nearly indistinguishable stylet. It is unknown how males migrate to the alimentary tract without a functional stylet, and one may speculate that males latch onto migrating females or use a communal wormhole to exit. Lacking a functional stylet, males are unable to parasitize a thrips host; supposedly, their only function in the free-living stage is to mate with females (Lysaght, 1936; Nickle and Wood, 1964). Although never observed, it has been suggested that mating occurs during the free-living stage (Lysaght, 1936; Nickle and Wood, 1964; Reddy et al., 1982). However, the risks of desiccation and failure to locate a female suggest that mating may occur inside the host. Aggregations of male and female nematodes formed in the hindgut suggest that males may inseminate females just prior to their emergence. This hypothesis is supported by observations that *T. fuscum* males emerge from the host later than females (Sims et al., 2005). Mating within the host insect has been reported for *Parasitylenchus*, an Allantonematid Tylenchid parasite of *Drosophila recens* (Perlman and Jaenike, 2001). Typically, Thripinema has a 19:1 (female: male) sex ratio, suggesting a tendency towards parthenogenesis in the absence of males (Sims et al., 2005). The infective free-living females, unlike the egg-producing females, have a cuticle similar to that of plant-parasitic nematodes. Under optimal conditions, free-living female and male *Thripinema* survive an average of 86 and 61 hours, respectively (Mason and Heinz, 2002). Survival of male and female nematodes after emergence is short due to unstable moisture and heat conditions (Mason and Heinz, 2002; Loomans et al., 1997).
Thripinema spp. have dramatic impacts on thrips populations in field conditions by reducing feeding and fecundity rates of females and subsequently decreasing TSWV spread. The potential of this nematode to act as a biological control agent has been evaluated, and all reports suggest the potential of using Thripinema spp. as inoculative agents against pest thrips in both greenhouse and field conditions (Arthurs and Heinz, 2002, 2003, 2005; Funderburk et al., 2002; Mason and Heinz, 2002; Lim and Van Driesche, 2004, 2005; Sims, 2003; Sims et al., 2005; 2009). In nature, the short-lived, free-living males and females are most commonly found in flower perianths, where thrips aggregate to mate and feed on pollen, and which are hypothesized to be the primary site for transmission (Crespi, 1993; Tipping et al., 1998; Sims et al., 2005). The timing underlying the spread of Thripinema in local thrips populations is not well understood. In groundnut, both the free-living nematode and the flowers are short-lived, mandating that the parasitized thrips remain in close proximity with healthy conspecifics.

Goals, Objectives, Hypotheses, and Expected Outcomes

The F. fusca/T. fuscum system is a unique and ideal model for examining the interface between a host insect and its obligate parasite. First, T. fuscum has a direct life cycle that does not require an intermediate host; therefore, all physiological development occurs inside the thrips. Second, unlike many other vertebrate nematodes that develop within host tissues, all Thripinema life stages exist in the hemocoel and can be readily collected. Third, Thripinema parasites depend upon their host for survival and transmission and therefore have negligible effects on thrips longevity or mortality (Sims et al., 2005). Fourth, a generation of Thripinema parasites can be completed in as few as nine days (Sims et al., 2005). Finally, F. fusca harbors numerous endoparasites, and the multitrophic relationship between them and their host provides different approaches for determining how Thripinema may influence host vector competence. Importantly, the proof of concept established for the F. fusca/T. fuscum model may be extended
to other thrips pests, such as viruliferous *F. occidentalis* parasitized by the entomogenous nematode *T. nicklewoodi*. The overall goal of this research project was to determine if, and to what extent, the obligate parasite *T. fuscum* influences the biology of *F. fusca* and its association with the entomoparasites *Wolbachia, P. ananatis*, and TSWV. The specific objectives to reach this goal are listed below.

In Chapter 2, I discuss utilizing a multigene approach using 16S rRNA, COI and MLST gene fragments to identify if one or more *Wolbachia* strains are associated with *F. fusca* and *T. fuscum* populations, and determine the influence of *Wolbachia* on *F. fusca* reproduction by comparing the development and reproductive biology (phenotype) of infected vs. antibiotically-cured host individuals. In Chapter 3, I use histological techniques to document the (1) the internal morphology of non-parasitized *F. fusca* adult females; (2) the life cycle of *T. fuscum* and provided a detailed *in vivo* account of this novel insect parasitic nematode including development, reproduction, and migration of the infectious female nematode and her progeny; and (3) compared and contrasted the impact of *T. fuscum* on non-parasitized and parasitized *F. fusca* females target tissues and cells. In Chapter 4, I discuss the effects of gender, age, wing form, viral infection, and nematode parasitism on the feeding behavior and TSWV transmission of *F. fusca*. In Chapter 5, I report the the impact of *T. fuscum* on *F. fusca* to harbor the plant pathogens *P. ananatis* and TSWV using quantitative biological, molecular, and histological approaches. Chapter 6 is a summary of the research findings and future directions.

The central hypothesis is that parasitism by *T. fuscum* alters the biology and reduces the entomopathogen content of its *F. fusca* host. The expected outcome of this project is to have a better understanding of how insect-parasitic nematodes interact with thrips *in vivo*, and subsequently, how parasites may suppress host vector competence. Deciphering the mechanisms
underlying the *in situ* interactions between the parasite, the host thrips, and associated entomoparasites is not the goal of this dissertation (but see Chapter 6 for potential mechanisms).

Results from this project may assist in identifying mechanisms that parasites/pathogens use to modulate the physiology of their respective insect vectors. For example, elucidating the mechanisms responsible for shutting off egg production in parasitized thrips may provide novel avenues for regulating the intrinsic rate of increase of this pest insect. Likewise, understanding the mechanism(s) leading to reduced Tospovirus competency (acquisition and transmission) in parasitized thrips also may provide targets to suppress the spread of disease. From a broader perspective, the information generated by these studies will enhance current understanding of host-parasite dynamics and potentially provide a basis to develop this unique biological control agent to better regulate this important insect vector.
Figure 1-1. The life cycle of *Frankliniella fusca* at 27°C. The number of days spent at each stage is in parentheses. (A) Egg embedded in plant tissue; (B–C) Larval stages; (D–G) Macropterous pupa and adults; (H–K) Brachypterous pupa and adults.
Figure 1-2. Illustrations of *Arachis hypogaea* and *Tomato spotted wilt virus*. (A) Healthy *A. hypogaea* plant; (B) *A. hypogaea* with TSWV symptoms; (C–D) *A. hypogaea* leaflet showing TSWV symptoms. Scale: (C) 10 mm; (D) 1 mm.
Figure 1-3. A collage of various fungi recovered from *Frankliniella fusca* laboratory and field populations in north central Florida.
Figure 1-4. Differential interference contrast (DIC) microscope images of *Entomophthorales* sp. stages infecting *Frankliniella fusca*. (A–B) Zygospores; (C–D) Conidia; (E–F) Conidiophores.

Figure 1-5. Scanning electron microscope images of *Entomophthorales* sp. stages infecting *Frankliniella fusca*. (A) *F. fusca* with a halo of primary conidia and a secondary saprophytic fungal infection; (B) Primary conidia; (C–D) Germinating conidia; (E–F) Conidiophores.
Figure 1-6. *Entomophthorales* sp. hyphal bodies in *Frankliniella fusca*. (A) A longitudinal thick section of a *Frankliniella fusca* female infected with an *Entomophthorales* sp.. Note the displacement of the thrips organs as a result of the abundance of hyphal bodies; (B) *Entomophthorales* hyphal bodies with measurements (26.7 µm × 8.1 µm); (C) Transmission electron micrograph of the hyphal bodies inside *F. fusca*. The hyphal bodies are binucleate with a poorly defined wall and contain numerous lipid droplets.
Figure 1-7. The progression of an *Entomophthorales* sp. infection in the female *Frankliniella fusca*. (A) Healthy *F. fusca* female; (B) *F. fusca* female exhibiting symptoms of an *Entomophthorales* sp. infection (note the swollen abdomen); (C) *Entomophthorales* sp. spores penetrating through the cuticle of the *F. fusca* female; (D) Side profile of *F. fusca* female with *Entomophthorales* sp. breaking the insect cuticle.
Figure 1-8. The life cycle of *Thripinema fuscum* in a female *Frankliniella fusca* host. (A) Adult *F. fusca* female; (B) Progressive enlargement of the parasitic female (right to left); (C) Eggs produced by the parasitic female; (D–F) First–through third–stage juveniles; (G) Infectious free-living females; (H) Free-living male. Ingress of (G) regenerates the cycle.
CHAPTER 2
MOLECULAR IDENTIFICATION OF THE REPRODUCTIVE PARASITE WOLBACHIA IN HOST-PARASITE POPULATIONS OF FRANKLINIELLA FUSCA AND THRIPINEMA FUSCUM

Introduction

The biology of *Wolbachia* is reviewed elsewhere (see Chapter 1). Currently the *Wolbachia* genus contains one species, *W. pipiensis*, with strain subdivisions representing eleven supergroups distributed throughout the Arthropoda and Nematoda phyla: arthropods (A-B), filarial nematodes (C-D), springtails (E), nematodes, arthropods, hexapods (F), spiders (G), termites (H), mites (I), (J) and (K) (Lo et al., 2002; Baldo et al., 2006; Bordenstein et al., 2005; Ros et al., 2009). Historically, many of these strains were placed in supergroups based on sequencing of the *Wolbachia* specific 16S rRNA (*wspec*) and surface protein (*wsp*) or cell division protein (*ftsZ*) genes (Zhou et al., 1998). However, this technique has been scrutinized for not adequately typing and quantifying strain diversities within a host because of extensive intragenic recombination and strong diversifying selection at the *wsp* gene (Werren and Bartos, 2001; Jiggins et al., 2001; Jiggens, 2002; Reuter and Keller, 2003; Baldo et al., 2002, 2005 2006; Paraskevopoulos et al., 2006; Baldo and Werren, 2007).

The recently developed multilocus sequence typing system (MLST), a process in which ~400-500 internal fragments of five conserved housekeeping genes (*gatB, coxA, hcpA, ftsZ*, and *fbpA*) are combined into a sequence type (ST) and the alleles at each locus are used as molecular markers to genotype a strain, is a more rigorous technique for annotating *Wolbachia* strains (Maiden et al., 1998; Urwin and Maiden, 2003; Paraskevopoulos et al., 2006; Baldo et al., 2006). Although not part of the MLST scheme, the *Wolbachia* surface protein (*wsp*) can be used as an additional marker to type strains by examining shuffling of a relatively conserved set of amino acid motifs within four hypervariable regions (HVR1-HVR4) of the gene (Jolley et al., 2004;
Baldo et al. 2006). The complete MLST strain typing system includes a web-accessible central database that allows for extensive comparative analyses between Wolbachia strains (Jolley et al., 2004; Baldo et al., 2006). This molecular multigene approach, in combination with biological phenotyping (see Chapter 1) and histological examination (see Chapter 3), currently offers the most reliable method for identifying and classifying Wolbachia strain(s) as well as deciphering the impact of Wolbachia on host biology.

The previously observed female-biased sex ratios of F. fusca and T. fuscum suggest Wolbachia may be dictating the reproductive biology of host and parasite populations (Sims et al., 2005). The objectives of this chapter were to (1) use a multigene approach to identify if one or more Wolbachia strains are associated with F. fusca and T. fuscum populations and (2) determine the influence of Wolbachia on F. fusca reproduction by comparing the development and reproductive biology (phenotype) of infected vs. antibiotically-cured host individuals. Findings are supported by electron microscopic examination targeting the reproductive tissues of non-parasitized and parasitized F. fusca and T. fuscum.

**Materials and Methods**

**Sample collection and DNA extraction:** To test for Wolbachia infection(s) in F. fusca and T. fuscum populations, male and female thrips were collected from the flowers of groundnut (Arachis hypogaea and A. glabrata) between March 2007 and November 2009 at two locations: (1) The Plant and Science Research Education Unit of University of Florida’s Institute of Food Agricultural Sciences facility at Citra in Marion County, FL (29°24’ N 82°10’ W), and (2) Fifield Hall of University of Florida's main campus at Gainesville in Alachua County, FL (29°38’ N 82°21’ W). A synopsis of the collection data is listed in Table 2-1. The excised flowers were placed into labeled Ziploc® bags and transported on ice to the laboratory for processing. In the laboratory, flowers were destructively sampled and female F. fusca collected
with an aspirator attached to a vacuum pump. Aspirated thrips were placed in 1.5 ml Eppendorf microcentrifuge tubes with a 1-cm peanut disc and maintained at 27°C and a 14 hour light period for 24 h. To determine parasitism status of the thrips, individuals were transferred to a new tube, the old tube was rinsed with 200 µl of water; and the rinsate was observed for free-living nematodes at 50X magnification. Thrips were stored as non-parasitized or parasitized cohorts (n=20 unless otherwise stated) in sterile 1.5 ml Eppendorf microcentrifuge tubes at -80°C. Free-living female and male \( T. fuscum \) were collected from recovered parasitized females, pooled, centrifuged, and stored in cohorts of ~500 in 100 µl of water at -80°C. A synopsis of the \( F. fusca \) and \( T. fuscum \) collection data including stage, gender, location, and date is listed below (Table 2-1). The number of cohorts used in each molecular analysis is listed accordingly in the results. DNA was extracted from the \( F. fusca \) and \( T. fuscum \) cohorts using the Epicentre MasterPure Yeast DNA Purification Kit (Epicentre Biotechnologies, Madison, WI) following the manufacturer’s protocol.

**Primer information, polymerase chain reaction (PCR), and sequencing:** A description of the genes and primer features are summarized in Table 2-2. All primer sequences and PCR conditions were kindly provided by Kostas Bourtzis (University of Ioannina, Greece). The primers for DNA amplification of the \( Wolbachia \) specific 16S rRNA gene (V6-V8) and \( wsp \) (HRV-1HRV4) were specifically designed to amplify variable and conserved regions within the genes, thus facilitating identification of \( Wolbachia \) infection(s) (Baldo et al., 2006; Petrosino et al., 2009). The five genes for MLST were selected based on the following conditions: (1) presence throughout the sequenced Rickettsiales, (2) a single copy in the \( Wolbachia \) genome, (3) a wide distribution in the \( Wolbachia \) genome, and (4) evidence of strong stabilizing selection within the \( Wolbachia \) genus (Jolley et al., 2004; Baldo et al., 2006). PCR amplification of all
genes was performed by using 1 µl of DNA, 2 µl of 10X reaction buffer, 1.8 µl of 25 mM MgCl2, 0.1 µl of dNTPs, 0.5 µl of forward primer (25 pmoles/µl), 0.5 µl of reverse primer, 0.1 µl of Taq polymerase, and 14 µl of sterile distilled water. The thermocycler (GeneAmp PCR System 2700, Applied Biosystems Foster City, CA) was programmed for an initial denaturing at 94°C for 5 m, followed by 34 cycles of 30 s at 94°C, 20 s at 55°C, and 90 s at 72°, one cycle of extension at 72°C for 10 m, and a final hold at 4°C. PCR products were run on a 1.2% agarose gel and stained with ethidium bromide to confirm amplification of the expected product. PCR products were purified (QIAquick PCR Purification Kit, QUIAGEN, Valencia, CA) and sent to the University of Florida’s Interdisciplinary Center for Biotechnological Research (UF-ICBR) for sequencing. Products were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and automated DNA sequencing (Applied Biosystems Model 3130 Genetic analyzer).

16S rRNA: Electropherograms obtained from the sequenced 16S rRNA PCR products were visually inspected, and the sequences were assembled into contigs and edited using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI). A nucleotide query was conducted on the edited sequences using the BLASTn database (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm product identity to the *Wolbachia* 16S ribosomal RNA gene. The edited 16S rRNA sequences were combined with an additional 27 partial and complete 16S rRNA sequences that were selectively chosen and represent a subset of the *Wolbachia* supergroups A-K (obtained from Kostas Bourtzis) (Appendix A1). The partial 16S sequence for *Escherichia coli* served as the outgroup. All 16S sequences were aligned using ClustalX 2.0 (Thompson et al., 1997), trimmed, and further edited in MacClade 4.0 as needed (Maddison and Maddison, 2000). Phylogenetic analyses were executed on the aligned sequences using the program PAUP 4.0b4a (Swofford,
2003). Phylogenetic trees were constructed using with distance analysis and neighbor-joining, along with additional default settings in PAUP. The robustness of the tree was tested using bootstrap and jackknife branch support values. A maximum of 1000 trees were searched for each data set followed by a 50% majority rule, strict consensus of the best fit trees.

**wsp typing:** Electropherograms obtained from the sequenced wsp PCR products were edited as described above and the primer sequences were marked. Individual nucleotide sequences were trimmed using a reference template from the wsp gene of wMel, a supergroup A Wolbachia strain from Drosophila melanogaster, located at the Wolbachia wsp typing protocol website (http://pubmlst.org/wolbachia/wsp/) (Jolley et al., 2004). A single nucleotide sequence query was conducted on each of the edited nucleotide sequences using the default settings. The resulting query indicated either a direct or partial match of the wsp locus to others in the database. If an exact match was not found, a basic local alignment search tool (BLAST) was performed to identify the nearest allele and provided the number of mismatches, gaps, and alignment to the closest allele. In addition, the generated wsp amino acid (AA) sequence was divided into four consecutive hypervariable regions flanked by highly conserved regions: HVR1 (AA 52 to 84), HVR2 (AA 85 to 134), HVR3 (AA 135-185), and HVR4 (AA 186 to 222) (Baldo et al., 2005). These regions were queried via BLAST to HVRs already in the database which provided a complete or partial match with similarities to the closest peptide(s). Alignments of the wsp AA profiles were generated using ClustalW2, a general purpose multiple sequence alignment program provided by the European Bioinformatics Institute (http://www.ebi.ac.uk/), to identify similarities and/or differences in each hypervariable region.

**MLST:** Non-parasitized F. fusca female populations that consistently provided a strong amplification signal from an initial screen of select genes were selected for the MLST (see
Electropherograms obtained from the sequenced *gatB, coxA, hcpA, ftsZ, and fbpA* PCR products were edited as described above. Sequences were trimmed by aligning each individual query sequence to a gene-specific polymorphic nucleotide map provided by the Wolbachia MLST database (http://pubmlst.org/wolbachia/) (Jolley et al. 2004). A multiple locus query was conducted on the trimmed sequences of all five genes for each cohort population. Samples with identical nucleotide sequences at a given locus were assigned the same allele number. For the samples with unique nucleotide sequences (*i.e.*, those not having an exact match to alleles in the database), the number of mismatches, gaps, and alignment to the closest allele was provided. The five MLST allele numbers for each cohort population were then combined and assigned an allele profile or sequence type (ST). The MLST profiles obtained for each cohort population were then visually compared to the 16S rRNA and *wsp* profiles to determine potential factors (*e.g.*, time, space, or parasitism) that may be driving Wolbachia nucleotide divergence in populations of *F. fusca* and *T. fuscum*.

**Effects of Wolbachia on *F. fusca* reproduction:** A series of laboratory bioassays were conducted to determine the effect of antibiotic treatment on the sex ratio of *F. fusca*. Thrips progeny from gene screen populations #13 and 14 (see Table 2-1) maintained in laboratory colonies were used for all the phenotype bioassays. For all treated groups, the antibiotic was offered by restricting *F. fusca* to the lid of a 1.5-ml microcentrifuge tube with parafilm and inverting a microcentrifuge tube containing an antibiotic solution (antibiotic + 10% honey in double distilled water) over the lid (herein referred to as the therapy chamber). A water solution (10% honey in double distilled water) was administered to the control groups. For each assay, an equal number of tubes without either solution were prepared to ensure *F. fusca* ingested the drinking water in the treatment therapy chambers as demonstrated by starvation of the confined
thrips in the empty tubes. Unless otherwise stated, all laboratory bioassays were conducted at 25°C and a 14 h light photoperiod.

The first bioassay tested the efficacy of four antibiotics against *Wolbachia* in *F. fusca*. Stock solutions of tetracycline, streptomycin, and ampicillin (50 mg/ml; Sigma) and kanamycin (25 mg/ml; Sigma) were stored at -20°C prior to use. The stock antibiotics were diluted with a 20% honey solution to give a final concentration of 25 mg/ml for tetracycline, streptomycin, and ampicillin, and 12.5 mg/ml for kanamycin. The antibiotic solutions were administered orally to female thrips for five consecutive days. The therapy chambers were refreshed daily with the appropriate solution (*i.e.*, antibiotic or water). After five days, females were removed from the enclosed lids of the therapy chamber and stored at -80°C. The experiment was replicated three times. DNA was extracted as described above and PCR was performed on DNA samples with *Wolbachia* specific 16S rRNA primers (*wspec*). The activity of each antibiotic against *Wolbachia* was determined visually by running the PCR product on a 1.2% agarose gel and staining with ethidium bromide.

The second bioassay tested the effect of *Wolbachia* on gender determination of larval *F. fusca*. The selected antibiotic was based on results from the prior experiment which provided the highest level of activity against *Wolbachia*. First instar *F. fusca* randomly collected from laboratory colonies (maintained as described in Chapter 2) were placed in cohorts (*n*=6) in the therapy chamber. After 48 h, larvae were removed from the lid of the therapy chamber and placed in a 1.5-ml microcentrifuge tube with a peanut leaflet. The thrips was placed in a new tube provisioned with a fresh peanut leaflet (1-cm²) daily until adult eclosion. The gender of each thrips was recorded. The experiment was replicated three times.
The third bioassay tested if the arrhenotokous parthenogenesis displayed by *F. fusca* is the result of *Wolbachia* infection(s). Non-choice mating experiments involving virgin and mated female *F. fusca* with and without antibiotic were designed. To collect virgin *F. fusca*, late stage pupae were randomly collected from laboratory colonies and placed individually into a 1.5-ml microcentrifuge tube provisioned with a 1-cm² peanut leaf disc. Eclosed adults were collected and placed into a therapy chamber (with or without antibiotic) for 48 h. Adults were removed from the therapy chambers and the females were placed in a new tube containing only a leaf disc (virgin) or a leaf disc plus a non-treated or treated male (mated) for 24 h. The following day, females were individually placed into a new 1.5-ml microcentrifuge tube provisioned with a leaf disc (1-cm²) and the males were discarded. The leaf discs were refreshed every 48 h for six days and the old leaves were stored for one week at 23°C and 14 h light period. After a one week incubation period, leaf discs were examined for emerging larvae for five days. When present, the larvae were transferred to a new 1.5-ml tube and provisioned with a fresh leaf disc daily until adult emergence. The number of progeny and gender derived from each female was recorded.

**Histology:** See Chapter 3 for the transmission electron microscopy protocol used for the histological examination of *Wolbachia* in individual non-parasitized and *T. fuscum* parasitized *F. fusca*.

**Results**

**16S rRNA:** A total of 44 DNA preparations were screened for infection(s) using *Wolbachia* specific 16S rRNA primers. Of these, 36 preparations representing samples from healthy *F. fusca*, parasitized *F. fusca*, and free-living *T. fuscum* populations were subjected to a gene screen. A moderate to strong amplification signal was detected in 30 of the 36 samples using the *Wolbachia* specific 16S rRNA primers (Figure 2-1A). Sequence data was obtained for 28 samples (Appendix A2). The 28 edited 16S rRNA sequences were subjected to a
phylogenetic analysis to determine relationships between the *F. fusca* and *T. fuscum* populations (Figure 2-2). The concatenated phylogenetic tree separated the samples into three distinct subgroups (A1, B1, and B2). Subgroup A1 contained six free-living *T. fuscum*, five non-parasitized female, and five parasitized female *F. fusca* cohort populations (n=16) and clustered into Supergroup A. The other two subgroups, B1 and B2, had high homology to Supergroup B. Subgroup B1 (n=10) was comprised of five non-parasitized female (n=5), two non-parasitized male (n=2), and three parasitized *F. fusca* female cohort populations. Subgroup B2 contained only 2 populations, one non-parasitized and one parasitized *F. fusca* female cohort. Free-living *T. fuscum* were not associated with subgroup B1 or B2. All healthy male populations clustered into subgroup A, and parasitized male populations clustered into subgroup B. A total of 11 of the 15 cohort populations associated with *T. fuscum* (either free-living or parasitized) were in subgroup A. Seven of the 13 healthy populations were clustered into subgroup B1.

**wsp typing:** A total of 36 of the 44 samples were selected for a gene screen using wsp primers. The wsp primers amplified 26 of the samples as demonstrated by a moderate to strong band by PCR detection (Figure 2-1B). Sequence data were obtained for 25 samples (Appendix A3). The edited wsp sequences of select non-parasitized *F. fusca* female cohorts (n=6) included all four hypervariable regions of the gene. Alignment of these sequences confirmed similarity of the gene across five samples (#2, 6, 7, 8, 20) with distinct regions of nucleotide divergence in the HVR1 (Figure 2-3). The wsp sequences from samples #2 and #6 were identical across the four conserved and hypervariable regions. Sequences #7, #8, and #20 were identical across HVR2-4 and CR3-4 but with distinct motifs in the HVR1 and CR2 regions. Sequence #11 had a high level of divergence from the other sequences in all hypervariable and conserved regions (motif in dark gray). Comparing similarities and differences in the wsp sequence data between the six
cohort populations mirrored differences found for the 16S rRNA phylogeny with respect to supergroup designation. The 16S rRNA phylogenetic tree grouped #2, 6, 7, 8 and 20 into subgroup B1 and #11 into subgroup B1. The wsp sequences for #2, 6, 7, 8, and 20 were highly homologous, whereas the wsp sequence for #11 had a high level of divergence from the other sequences.

**MLST:** A gene screen was conducted on 36 of the 44 samples (Figure 2-1C-G). There were 17 non-parasitized female *F. fusca*, 10 parasitized *F. fusca*, and nine free-living *T. fuscum* in the analysis. Amplification from all samples was most successful with the coxA and ftsZ primers with a visible band detected by conventional PCR for 31 and 30 of the samples, respectively (Figure 2-1C, F; Table 2-4). The gatB and ftbA primers successfully amplified *Wolbachia* in 19 and 12, respectively, of the samples (Figure 2-1E, G; Table 2-4). *Wolbachia* was detected in only 8 of the samples using the hcpA primers (Figure 2-1D). Overall, the MLST primers successfully amplified product from 61, 62, and 38% of the non-parasitized *F. fusca* females, parasitized *F. fusca* females, and free-living *T. fuscum* cohorts, respectively (Table 2-4). *Wolbachia* was not detected by conventional PCR in two samples (non-parasitized *F. fusca* females (#39) and *T. fuscum* (#40)).

Six non-parasitized *F. fusca* cohort populations consistently provided a strong amplification signal in the initial gene screen as demonstrated by the presence of a band after detection by conventional PCR. Partial and complete MLST sequence data was obtained for four and six of the populations, respectively (product was not detected in sample #11 using the hcpA primers and in sample #20 using the fpbA primers) (Table 2-5; Appendix A4). Comparisons across samples indicated minor nucleotide divergences across loci, with sample #11 having the highest level of divergence. There was 100% homology to gatB in five of the six
samples and to coxA in three of the six samples. All other alleles were unique. Two ST were identified for the samples with complete MLST sequence data. Samples #7, 2, and 6 share one ST and sample 8 differs in ST only by allele 15 for fbpA. The variance at alleles between the four ST is at the same base nucleotide.

**Effects of Wolbachia on F. fusca reproduction:** A range of antibiotics were chosen that were expected to vary in their activity against Wolbachia. Of the four antibiotics tested, ampicillin, streptomycin, and tetracycline (50 mg/ml) showed good activity against Wolbachia. Kanamycin (25 mg/ml) did not have any activity (Figure 2-4). Visually, tetracycline had the greatest impact against Wolbachia as detected by conventional PCR and was the selected antibiotic for the phenotyping bioassays.

First instar F. fusca were treated with tetracycline (50 mg/ml) for 48 h (n=174) and their gender recorded at adult eclosion. A total of 29 trials were conducted and each trial included three control and three tetracycline-treated larval F. fusca. Three control thrips and eight treated thrips died at this concentration of antibiotic during the assay. The gender was recorded from 84 control thrips and 79 tetracycline-treated thrips (Table 2-6). Larval thrips fed a liquid honey solution (=Wolbachia infected) emerged predominantly as females (66%), for a female to male ratio of 2 to 1. Larval thrips fed a liquid antibiotic solution (=Wolbachia-cured) emerged predominantly as males (61%), for a female to male ratio of 0.64 to 1.

The last two bioassays examined the effects of removing Wolbachia by antibiotic treatment on offspring production of virgin and mated F. fusca females. A total of five trials were conducted for each assay and each trial included three control and three tetracycline-treated adult F. fusca females. The virgin control females (n=15) produced an average of 4.67 ± 2.87 eggs (with a range of 0 - 12 total eggs per female) and the virgin treated females (n=15)
produced an average of 4.07 ± 2.7 eggs (with a range of 0 to 10 total eggs per female). All of the eggs in both treatments emerged as males after adult eclosion. The mated control females (n=15) produced an average of 4.47 ± 2.56 eggs (with a range of 0 to 11 total eggs per female). The mated treatments (n=14) produced an average of 1.86 ± 1.23 eggs (with a range of 0 to 4 total eggs per female). The female to male sex ratio of progeny from the mated control females was 2 to 1 (45 females and 22 males) and the progeny from mated treated females were 100% male (n=26).

**EM:** Transmission electron micrographs of thin sections from non-parasitized and *T. fuscum* parasitized *F. fusca* females revealed the presence of numerous small (~1 µm) bacteria in the yolk of the oocytes and the cytoplasm of follicle cells surrounding the oocyte (Figure 2-5). These bacteria were not observed in any other *F. fusca* tissues.

**Discussion**

Use of the full MLST gene set for strain typing indicates that non-parasitized *F. fusca*, parasitized *F. fusca*, and free-living *T. fuscum* are all associated with one or more *Wolbachia* strains. *Wolbachia* has been detected in other arrhenotokous and thelytokous thrips populations and in a plant parasitic nematode (see Chapter 1; Araki et al., 1999; Pintureau et al., 1999; Haegeman et al., 2009; Kumm and Moritz, 2009). To my knowledge, this is the first report of (1) multilocus sequence typing and supergroup designation of a *Wolbachia* strain(s) associated with a Thysanopteran and (2) detection of *Wolbachia* in an insect parasitic nematode. Horizontal transfer of *Wolbachia* has been documented between a host and parasite (see Chapter 1), but further analyses need to be conducted before concluding lateral transmission between the two taxa occurred. Interestingly, the *F. fusca* 16S rRNA sequence data was highly homologous to sequence data obtained from *Bryobia* sp. (Figure 2-2). These sap-feeding mites are commonly found on peanut and have been recovered numerous times from peanut plants maintained in the
greenhouse for *F. fusca* colony rearing. Horizontal transfer of *Wolbachia* may also have occurred between *Bryobia* sp. and *F. fusca*.

Phylogenetic analysis of the 16S rRNA revealed two major groupings of *Wolbachia* in the cohort populations. The presence of *T. fuscum* appears as the dominant factor correlated with the subgroup segregation in the cohort populations. The majority of non-parasitized *F. fusca* clustered into Supergroup B (8/12) whereas the parasitized *F. fusca* and free-living *T. fuscum* grouped into Supergroup A (11/16). These data suggest, but do not conclude, parasitism may induce a switch in the dominant *Wolbachia* strain infecting *F. fusca*. The cause of this switch is unknown. Initially, I suspected the *Wolbachia* strain(s) associated with the *T. fuscum* nematode may be outcompeting (=destroying) the strain found in the non-parasitized *F. fusca*. As reviewed in Chapter 1, *Wolbachia* is considered an obligatory mutualist in some insects and is required for successful oogenesis. This outcompetition, or killing of host *Wolbachia*, could potentially be the cause for the observed sterility in parasitized *F. fusca*. However, antibiotically treated or *Wolbachia*-cured females are still able to reproduce and there was not 100% homology in 16S sequence data between the *T. fuscum* and parasitized *F. fusca* population cohorts. An alternative hypothesis proposes the competitive intraspecific exclusion of *Wolbachia* strains within a parasitized *F. fusca*. *Wolbachia* is predominantly found in the reproductive tissues of infected individuals but can also be associated with somatic tissue (Min and Benzer, 1997; Dobson et al., 1999; Cheng et al. 2000). Healthy *F. fusca* have polymorphisms in the 16S rRNA electropherograms, suggesting multiple *Wolbachia* strains within the examined population cohorts (data not shown) - one associated with the reproductive system and one systemically infecting the somatic tissues. As reviewed in Chapters 1 and 2, *T. fuscum* causes a degeneration of the ovaries in parasitized *F. fusca*. This degeneration may reduce the number of bacteria
associated with the reproductive tissues to non-detectable levels, and as a result, the Wolbachia specific primers were only able to detect the underlying systemic strain. However, thorough histological examination did not detect Wolbachia symbionts in tissues other than those associated with the reproductive system of F. fusca. Bacterial associations with F. fusca individuals were restricted to the midgut and ovaries (see Chapters 3 and 5).

In the present study, Wolbachia influences the reproductive biology of the arrhenotokous F. fusca. Wolbachia did not alter the number of eggs or gender of offspring from virgin F. fusca, but increased the fertility and production of female progeny in mated F. fusca. Additionally, the presence of Wolbachia in larval F. fusca increases the number of females upon adult eclosion. As mentioned earlier, Wolbachia induces a variety of effects to host reproduction including cytoplasmic incompatibility, parthenogenesis, feminization, and male-killing (as reviewed in Werren, 1997). To my knowledge, there is no information in the literature regarding the influence of Wolbachia on arrhenotokous populations of haplodiploid insects. Haplodiploid thylytokous populations of wasps revert to male production when antibiotically cured of Wolbachia (Legner, 1985; Stouthamer and Luck, 1991; Zchori et al., 1992). In my study, Wolbachia-infected females produced twice as many eggs as Wolbachia-cured mated females. These results suggest Wolbachia infection may induce male-killing in the embryo of fecund F. fusca.

The influence of Wolbachia on the host-parasite dynamics of F. fusca and T. fuscum are unknown. Parasitism rates under field conditions are time-dependent and suggest extrinsic factors (e.g., temperature, humidity, rainfall, etc.) are critical for determining parasite success (data not shown). Parasitism rates in the laboratory are also time-dependent (e.g., laboratory parasitism rates from June to November increase from 25% to 85%) which suggests intrinsic
factor(s) (e.g., physiological regulation by host or parasite) may also influence parasitism rates (data not shown). These physiological factors may be directly or indirectly related to Wolbachia infection. For example, Wolbachia infection upregulates Aedes aegypti immune system and significantly inhibits the ability of Brugia filarial nematodes to develop inside mosquitoes (Kambris et al., 2009). Potentially, Wolbachia infection in the thrips host upregulates immune factors that are active against T. fuscum. Effects of these host immune factors on other entomoparasites (e.g., Pantoea ananatis and TSWV) in the F. fusca vector are discussed in Chapter 5. Clearly, more studies are needed to understand the interaction between Wolbachia and its F. fusca host and results from this chapter open avenues for new and unique management strategies to control thrips vector populations in agroecosystems (see Chapter 6).
Table 2-1. Collection data including cohort population, locality, and date labeled for *Wolbachia* gene screen and 16S rRNA analysis.

<table>
<thead>
<tr>
<th>Gene Screen #</th>
<th>16S Sample #</th>
<th>Cohort population</th>
<th>Collection locality</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>1</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>Unknown</td>
<td>2007</td>
</tr>
<tr>
<td>34</td>
<td>2</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>UF Campus</td>
<td>2008</td>
</tr>
<tr>
<td>40</td>
<td>3</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>UF Campus</td>
<td>2008</td>
</tr>
<tr>
<td>41</td>
<td>4</td>
<td>Parasitized <em>F. fusca</em> females</td>
<td>UF Campus</td>
<td>2008</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>Free-living <em>T. fuscum</em></td>
<td>UF Campus</td>
<td>2008</td>
</tr>
<tr>
<td>42</td>
<td>6</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>Citra (ornamental)</td>
<td>Oct-08</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>Citra (ornamental)</td>
<td>Oct-08</td>
</tr>
<tr>
<td>31</td>
<td>8</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>Citra (field)</td>
<td>Oct-08</td>
</tr>
<tr>
<td>44</td>
<td>9</td>
<td>Parasitized <em>F. fusca</em> females</td>
<td>Citra (field)</td>
<td>Oct-08</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>Free-living <em>T. fuscum</em></td>
<td>Citra (field)</td>
<td>Oct-08</td>
</tr>
<tr>
<td>1</td>
<td>11</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>UF Campus (first generation from colony)</td>
<td>May-09</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>UF Campus (first generation from colony)</td>
<td>May-09</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>Non-parasitized <em>F. fusca</em> females</td>
<td>UF Campus (first generation from colony)</td>
<td>May-09</td>
</tr>
<tr>
<td>7</td>
<td>14</td>
<td>Non-parasitized <em>F. fusca</em> males</td>
<td>UF Campus (first generation from colony)</td>
<td>May-09</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>Parasitized <em>F. fusca</em> females</td>
<td>Citra (field)</td>
<td>Aug-09</td>
</tr>
<tr>
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<td>16</td>
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<td>Citra (field)</td>
<td>Aug-09</td>
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<tr>
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<td>17</td>
<td>Parasitized <em>F. fusca</em> females</td>
<td>Citra (field)</td>
<td>Aug-09</td>
</tr>
<tr>
<td>8</td>
<td>18</td>
<td>Free-living <em>T. fuscum</em></td>
<td>Citra (field)</td>
<td>Aug-09</td>
</tr>
<tr>
<td>9</td>
<td>19</td>
<td>Free-living <em>T. fuscum</em></td>
<td>Citra (field)</td>
<td>Aug-09</td>
</tr>
<tr>
<td>13</td>
<td>20</td>
<td>Non-parasitized <em>F. fusca</em> females*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
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<td>21</td>
<td>Non-parasitized <em>F. fusca</em> females*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>17</td>
<td>22</td>
<td>Parasitized <em>F. fusca</em> females*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>18</td>
<td>23</td>
<td>Non-parasitized <em>F. fusca</em> males*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>19</td>
<td>24</td>
<td>Parasitized <em>F. fusca</em> males*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>21</td>
<td>25</td>
<td>Parasitized <em>F. fusca</em> males*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>23</td>
<td>26</td>
<td>Parasitized <em>F. fusca</em> pupae*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>24</td>
<td>27</td>
<td>Free-living <em>T. fuscum</em></td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>25</td>
<td>28</td>
<td>Free-living <em>T. fuscum</em></td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
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</tbody>
</table>
Table 2-1. Continued.

<table>
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<tr>
<th></th>
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<th>Non-parasitized <em>F. fusca</em> females*</th>
<th>Citra (field) (first generation from colony)</th>
<th>Nov-09</th>
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</tr>
<tr>
<td>15</td>
<td>-</td>
<td>Parasitized <em>F. fusca</em> females*</td>
<td>Citra (field) (first generation from colony)</td>
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</tr>
<tr>
<td>20</td>
<td>-</td>
<td>Parasitized <em>F. fusca</em> males*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>Non-parasitized <em>F. fusca</em> pupae*</td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
</tr>
<tr>
<td>26</td>
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<td>Free-living <em>T. fuscum</em></td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
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<tr>
<td>27</td>
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<td>Free-living <em>T. fuscum</em></td>
<td>Citra (field) (first generation from colony)</td>
<td>Nov-09</td>
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<tr>
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<td>Non-parasitized <em>F. fusca</em> females</td>
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</tr>
<tr>
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<td>Non-parasitized <em>F. fusca</em> females</td>
<td>Citra (field)</td>
<td>Oct-08</td>
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<tr>
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<td>Free-living <em>T. fuscum</em></td>
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<tr>
<td>43</td>
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<td>Non-parasitized <em>F. fusca</em> females</td>
<td>Citra (field)</td>
<td>Oct-08</td>
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</table>
Table 2-2. 16S, wsp, and MLST loci and primer features (taken from Baldo et al., 2006).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Designation</th>
<th>Primer Sequence (5’-3’)</th>
<th>Gene length (bp)</th>
<th>Amplified nt range (bp)</th>
<th>Fragment size (bp)</th>
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<td>Wolbachia specific 16SrRNA</td>
<td>WspecF</td>
<td>YATACCTATTCCGAAGGGGATAG AGCTTCGAGTGAAACCAATTCTGCAATARSTGATGARGAAAC</td>
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<td></td>
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<td></td>
<td>WspecR</td>
<td></td>
<td></td>
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<tr>
<td>wsp</td>
<td>Wolbachia surface protein</td>
<td>wsp_F1</td>
<td>CYGCACCAAYAGYRCTRTAAA</td>
<td>714</td>
<td>85-688</td>
<td>546</td>
</tr>
<tr>
<td></td>
<td></td>
<td>wsp_R1</td>
<td></td>
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<tr>
<td>gatB</td>
<td>aspartyl/glutamyl-tRNA(Gln) amidotransferase, subunit B</td>
<td>gatB_F1</td>
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<td>421-891</td>
<td>369</td>
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<td></td>
<td>gatB_R1</td>
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<tr>
<td>coxA</td>
<td>cytochrome c oxidase, subunit I</td>
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<td>491-977</td>
<td>402</td>
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<tr>
<td>hcpA</td>
<td>conserved hypothetical protein</td>
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<td>91-605</td>
<td>444</td>
</tr>
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<td>hcpA_R1</td>
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<tr>
<td>ftsZ</td>
<td>cell division protein</td>
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<td>435</td>
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<td></td>
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<td>ftsZ_R1</td>
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<tr>
<td>fbpA</td>
<td>fructose-bisphosphate aldolase</td>
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<td>GCTGCTCCRCTTGGYWTGAT</td>
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<td>429</td>
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<td></td>
<td>fbpA_R1</td>
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### Table 2-3. *Wolbachia* surface protein (*wsp*) loci and hypervariable region profile (HVR1-4) for the six non-parasitized *Frankliniella fusca* samples selected for MLST.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Locus</th>
<th>wsp</th>
<th>HVR1</th>
<th>HVR2</th>
<th>HVR3</th>
<th>HVR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td></td>
<td>372, 78</td>
<td>1</td>
<td>12, 71</td>
<td>21, 109</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(91.91%)</td>
<td>(90.01%)</td>
<td>(94.00%)</td>
<td>(96.08%)</td>
<td>(84.62%)</td>
</tr>
<tr>
<td>20</td>
<td></td>
<td>424, 296, 162</td>
<td>157, 17, 95,</td>
<td>17</td>
<td>88, 3</td>
<td>2, 109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(90.00%)</td>
<td>(60.61%)</td>
<td>(100%)</td>
<td>(98.04%)</td>
<td>(96.43%)</td>
</tr>
<tr>
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<td>424, 296, 162</td>
<td>88, 65</td>
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<td>88, 3</td>
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<tr>
<td></td>
<td></td>
<td>(94.38%)</td>
<td>(84.85%)</td>
<td>(100%)</td>
<td>(98.04%)</td>
<td>(96.43%)</td>
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<td>424, 296, 162</td>
<td>88, 65</td>
<td>17</td>
<td>88, 3</td>
<td>2, 109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(92.50%)</td>
<td>(75.76%)</td>
<td>(100%)</td>
<td>(98.04%)</td>
<td>(96.43%)</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>424, 296, 162</td>
<td>2, 77</td>
<td>17</td>
<td>88, 3</td>
<td>2, 109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(96.88%)</td>
<td>(93.94%)</td>
<td>(100%)</td>
<td>(98.04%)</td>
<td>(96.43%)</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>424, 296, 162</td>
<td>2, 77</td>
<td>17</td>
<td>88, 3</td>
<td>2, 109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(96.88%)</td>
<td>(93.94%)</td>
<td>(100%)</td>
<td>(98.04%)</td>
<td>(96.43%)</td>
</tr>
</tbody>
</table>

The number in parentheses represents the percent match to the alleles listed for each sample.

### Table 2-4. The percentage of each *Frankliniella fusca* cohort population with a visible amplicon of each primer by conventional PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Amplicon detected in sample</th>
<th>Non-parasitized female <em>F. fusca</em> (17)</th>
<th>Parasitized female <em>F. fusca</em> (10)</th>
<th>Free-living <em>T. fuscum</em> (9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S</td>
<td></td>
<td>88.24% (15)</td>
<td>90.00% (9)</td>
<td>66.67% (6)</td>
</tr>
<tr>
<td>wsp</td>
<td></td>
<td>82.35% (14)</td>
<td>90.00% (9)</td>
<td>44.44% (4)</td>
</tr>
<tr>
<td>coxA</td>
<td></td>
<td>82.35% (14)</td>
<td>90.00% (9)</td>
<td>88.88% (8)</td>
</tr>
<tr>
<td>hcpA</td>
<td></td>
<td>29.41% (5)</td>
<td>20.00% (2)</td>
<td>11.00% (1)</td>
</tr>
<tr>
<td>fibA</td>
<td></td>
<td>52.94% (9)</td>
<td>30.00% (3)</td>
<td>0.00% (0)</td>
</tr>
<tr>
<td>fisZ</td>
<td></td>
<td>82.35% (14)</td>
<td>100% (10)</td>
<td>66.67% (6)</td>
</tr>
<tr>
<td>gatB</td>
<td></td>
<td>58.82% (10)</td>
<td>70.00% (7)</td>
<td>22.22% (2)</td>
</tr>
<tr>
<td>Total %</td>
<td></td>
<td>61.18%</td>
<td>62.00%</td>
<td>37.78%</td>
</tr>
</tbody>
</table>

The number in parentheses represents the total number for each category.
Table 2-5. Allelic profiles of the six non-parasitized *Frankliniella fusca* samples selected for MLST.

<table>
<thead>
<tr>
<th>Sample</th>
<th>gatB</th>
<th>coxA</th>
<th>hcpA</th>
<th>ftsZ</th>
<th>fbpA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>43 (6)</td>
<td>32 (40)</td>
<td>-</td>
<td>6 (7)</td>
<td>15 (6)</td>
</tr>
<tr>
<td>20</td>
<td>39</td>
<td>14 (1)</td>
<td>6 (2)</td>
<td>7 (1)</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>14</td>
<td>6 (2)</td>
<td>7 (1)</td>
<td>9 (2)</td>
</tr>
<tr>
<td>8</td>
<td>39</td>
<td>14</td>
<td>6 (2)</td>
<td>7 (1)</td>
<td>15 (5)</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>14</td>
<td>6 (2)</td>
<td>7 (1)</td>
<td>9 (2)</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>14 (1)</td>
<td>6 (2)</td>
<td>7 (1)</td>
<td>9 (2)</td>
</tr>
</tbody>
</table>

The number in parentheses represents the number of nucleotide differences to the closest allele. A dash (-) designates sequence data was not obtained for the listed gene.

Table 2-6. The percentage of male and female *Frankliniella fusca* at adult eclosion after tetracycline (50 mg/ml) treatment as first instars.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Female</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (84)</td>
<td>66% (56)</td>
<td>33% (28)</td>
</tr>
<tr>
<td>Tetracycline-treated (79)</td>
<td>39% (31)</td>
<td>61% (48)</td>
</tr>
</tbody>
</table>

The number in parentheses represents the number for each treatment.
Figure 2-1  An initial *Wolbachia* gene screen of 36 *Frankliniella fusca* and *Thripinema fuscum* populations.  (A) 16S rRNA;  (B) *wsp*;
Figure 2-1 (continued). (C) coxA; (D) hcpA, (E) ftbA; (F) ftsZ; and (G) gatB primers. A star (*) designates samples with a weak to no signal present.
Figure 2-2. Neighbor-joining phylogenetic tree based on 16S rRNA nucleotide alignment. *Wolbachia* strains are characterized by the names of their host species. Names in bold represent cohort populations in the dataset with the numbers in parentheses corresponding to those listed in Table 2-1. The supergroup designations are shown on the right. Bootstrap and jackknife values are depicted above and below the branches, respectively.
Figure 2-3. Amino acid alignment of six \textit{wsp} sequences from non-parasitized female \textit{Frankliniella fusca} cohort populations. The six samples used for the alignment were the same samples selected for the MLST. Amino acid motifs at each hypervariable region (HVR1-4) are grouped by color according to their similarity to other sequences.
Figure 2-4. Conventional PCR detection of *Wolbachia* in *Frankliniella fusca* with 16S rRNA primers (wspec) after antibiotic therapy. (L) Marker Hyperladder II (5 µl); (1) 50 *F. fusca* from laboratory colony (+ control); (2) Ampicillin (50 mg/ml); (3) 10 *F. fusca* from laboratory colony (+ control); (4) Kanamycin (25 mg/ml); (5) Streptomycin (50 mg/ml); (6) Tetracycline (50 mg/ml).
Figure 2-5. Transmission electron micrographs of intracellular bacteria in the reproductive structures of non-parasitized and *Thripinema fuscum* parasitized *Frankliniella fusca* females. (A) Ovariole of a non-parasitized *F. fusca* parasitized individual with bacteria; (B) Magnification of the bacteria in the cytoplasm of the oocyte and surrounding follicle cell; (C) Ovariole of a *T. fuscum* parasitized *F. fusca* female with bacteria; (D–F) Progressive magnification of (C).
CHAPTER 3
HISTOLOGICAL EXAMINATION OF FRANKLINIELLA FUSCA AND THRIPINEMA FUSCUM

Introduction

There is very limited information available in the literature on the internal anatomy and morphology of *F. fusca* despite this thrips importance as a vector of *Tomato spotted wilt virus* (TSWV, Bunyaviridae: Tospovirus) and *Pantoea ananatis* (Eubacteriales: Enterobacteriaceae) (Sakimura, 1969; Heming, 1970a,b; Wells et al., 2002; Gitaitis et al., 2003). Most histological studies conducted on Thysanoptera have been restricted to the *F. occidentalis* vector (Dallai et al., 1991, 1996, 1997; Del Bene et al., 1991; Hunter and Ullman, 1989, 1994; Kumm, 2002; Ullman et al., 1989). However, thrips morphologies vary greatly between species and may delineate persistent vectors from semi-persistent vectors and non-vectors (Ullman et al., 1989). A better understanding of *F. fusca* vector biology is essential for developing strategies to manage this crop pest.

In addition, there have been few studies pictorially documenting the *in vivo* life cycle of Allantonematidae nematodes and the pathological changes these parasites induce to host tissues (Schmidt and Platzer, 1980; Tomalak et al., 1984, 1988). The potential exists for *T. fuscum* to be commercialized as a natural enemy of *F. fusca*, but the *in situ* relationship between the parasite and host thrips has not been investigated. Therefore, the objective of this chapter was to conduct a histological examination and (1) document the internal morphology of healthy *F. fusca* females, particularly select tissues and cells affecting vector competence and reproduction; (2) examine the life cycle of *T. fuscum* including *in vivo* location and development of the parasitic female and juveniles, reproduction, and migration of late stage juveniles out of the thrips host; and (3) identify changes to host thrips target tissues and cells resulting from *T. fuscum* invasion and replication. Information gathered from these studies may provide insight into how the
parasitic *T. fuscum* alters host morphology and how such alterations influence *F. fusca* vector competence, and as a result, offer new strategies for controlling pest insect vectors.

**Materials and Methods**

**Specimen collection:** Female *F. fusca* were collected from peanut flowers (*Arachis hypogaea* L.) in Marion Co., Florida at the Institute of Food and Agricultural Science Plant Research and Education Unit (29°24’ N 82°10’ W). Specimens were selected from established non-parasitized and *T. fuscum* parasitized *F. fusca* laboratory colonies maintained as previously described (Sims et al., 2005).

**Light microscopy:** Non-parasitized and parasitized *F. fusca* females were dissected in a droplet of double distilled water on a glass slide using minuten pins. Dissected specimens were photographed with the Auto-Montage Pro System, a program which uses a series of captured images focused at different heights to form a high quality focused composite (Auto-Montage Pro 5.02.0096; Syncroscopy, Frederick, MD, USA). Free living *T. fuscum* were collected by rinsing 1.5-ml microcentrifuge tubes that previously held parasitized *F. fusca* adult females with 200 µl of Heps-buffered saline (pH 6.8). The buffer from each tube was collected and pooled into a single tube, centrifuged at 5,000g for 3 minutes, and exchanged with warm (~37°C) 2.5% gluteraldehyde buffered in 0.1M sodium cacodylate containing CaCl₂ (1 mg/ml). The recipes used for histology (i.e., fixative, buffers, post-fixatives, etc.) are listed elsewhere (Appendix B). The *in vivo* stages of *T. fuscum* were collected by dissecting fully parasitized *F. fusca* females in a 10 µl droplet of Heps-buffered saline. The droplet of nematodes was transferred into a 1.5 ml microcentrifuge tube containing warm fixative for 10 minutes. Nematodes were then placed individually in a droplet of glycerin, covered with a cover slip, and photographed under a differential interference contrast microscope with a RT Spot Diagnostics Imaging System (Spot Imaging Solutions, Sterling Heights, MI).
**Scanning electron microscopy**: Adult *F. fusca* females were immobilized on ice and adhered to double-sided tape attached to a microscope slide. A 10 µl droplet of warm fixative was immediately placed over the specimen. The submerged insect was pierced with a finely pulled capillary tube through the cuticle of each body segment to allow for the exchange of fluids. After five minutes, the specimen was transferred to a 1.5-ml microcentrifuge tube with warm, fresh fixative for two hours. Stages of *T. fuscum* were collected as described above and fixed for two hours at room temperature. Fixed specimens were transferred to a specimen holder that sandwiches microorganisms between nucleopore filters (0.4-µm; Nucleopore Corp., Pleasanton, CA) to reduce elimination of samples during the multiple fluid transitions required for processing (Kurtzman *et al.*, 1974). Using a syringe, samples were washed, post-fixed, and dehydrated (Table 3-1). Following the final dehydration in acetone, the syringe was removed and the specimens were dried using the critical point method (Bal-Tek 030 CPD, Cheshire, UK). The specimen holder was then disassembled and the nucleopore filters holding either *F. fusca* females or *T. fuscum* were attached to SEM stubs with double-sided adhesive. To process parasitized *F. fusca* females for SEM, individuals were fractured on a copper stub adhesive by lightly dragging a razorblade over the surface of the insect and prying open the cuticle using fine forceps and minuten pins. All stubs were coated with gold for 130 s in a sputter-coater (Denton Vacuum Desk II (Au/Pd), Moorestown, NJ, USA) and examined in a Scanning Electron Microscope (Hitachi FE-S4000, Hitachi High Technologies America, Illinois, USA) at 10 kV. Measurements from digital images were taken with the Quartz PCI Image Management System (Vancouver, Canada). It is important to note that shrinkage resulting from fixation typically results in a 30% size reduction of specimens and the reader should see Tipping *et al.* (1998) for exact measurements of *T. fuscum* stages.
Transmission electron microscopy: *Frankliniella fusca* females and *T. fuscum* individuals were prepared for transmission electron microscopy following the protocol described in Table 3-1. When needed, sodium bis (2-ethylhexyl) sulfosuccinate (Aerosol O.T. 100%) was used to break the surface tension and help submerge thrips in fixative. All samples were embedded in a 1% agarose block prior to post-fixation. Dehydrated samples were infiltrated with Epon-Araldite resin (cat no. 13940, EMS; Hatfield, PA, USA) and Z-60-40 embedding primer (cat no. 50440-10, EMS) was added to help the resin adhere to insect cuticle. Samples were placed in plastic molds and polymerized in a 65°C oven. Resin blocks were sectioned using a Reichert-Jung Ultracut E Microtome. Thick sections (0.5-µm) were stained with 1% toluidine blue in 1% borax for 15 s, covered with Permount and a cover slip, and viewed under a compound microscope. Images were collected with the RT Spot Diagnostics Imaging System. Ultrathin sections (70-90 nm) were taken with a diamond knife (Diatome; Hatfield, PA, USA), and collected on Formvar Carbon Coated Copper Grids (200 mesh; EMS, Hatfield, PA, USA). Sections were post-stained in 0.5% uranyl acetate for 10 minutes followed by Reynold’s lead citrate for 5 minutes and viewed at 75 kV with a Hitachi H-600 electron microscope.

**Results**

**Healthy female *F. fusca*:** Results herein are concordant with the few histological studies available in the literature that document the internal morphology of *F. fusca* and *F. occidentalis* (Heming, 1970; Ullman et al., 1989; De Bene et al., 1991; Dallai et al., 1991, 1996; Hunter and Ullman, 1992, 1994; Kumm, 2002). Externally, the head holds the antennae, compound eyes, ocelli, and piercing sucking mouthparts in a hypognathous position (Figure 3-1, Figure 3-2). Internally, the head contains the brain, ganglia, and nerves. The thorax, the second body segment, encloses the ovoid salivary glands and the anterior foregut. In addition, muscles predominate in the thoracic tagma and serve as points of attachment for the external wing and leg.
appendages. *Frankliniella fusca* have macropterous and brachypterous wing forms (Figure 3-3). The abdomen, the largest of the three body segments, is comprised of 13 segments that enclose the alimentary tract and reproductive structures. A saw-like ovipositor is present on the ninth segment (Figure 3-4). Abdominal fat body and muscles underlie the female thrips cuticle. A thick section of a healthy *F. fusca* female taken at 400X exhibits the location and fine detail of these structures (Figure 3-5). Herein, the internal anatomy of *F. fusca* will be briefly described according to those systems and/or tissues most affected by *Thripinema fuscum* parasitism (i.e., invasion, replication, and emergence): the digestive system, the reproductive system, fat body and muscle.

The digestive system of *F. fusca* consists of the salivary glands, foregut, midgut, hindgut, and Malpighian tubules. These organs flank the surrounding muscle tissue in the thoracic and abdominal tagma. The *F. fusca* salivary glands are comprised of two morphologically distinct types: short, ovoid glands that lie proximally to the esophagus in the thoracic region and long, tubular glands that extend from the ovoid glands to the anterior portion of the midgut (Figure 3-6A-B). The salivary glands produce and release saliva used for digesting food. The glands are endodermal in origin and easily recognizable under the electron microscope by the presence of cells containing spheroidal secretory granules and a thin, dense cuticle-lined lumen (Del Bene et al. 1999) (Figure 3-6C-D). Importantly, the ovoid salivary glands are the principal site for TSWV replication (Figure 3-7). The foregut (stomodaeum) sits immediately posterior to the salivary glands in the thoracic tagma and is the primary organ for ingestion. The foregut develops from invaginating ectodermal tissue and consists of a thin layer of epithelial cells lined by cuticular intima (Figure 3-8A-B). The midgut, located posterior to the foregut, is the principal site for secretion of enzymes and digestion and absorption of food particles (Figure 3-
The *F. fusca* midgut is convoluted and forms three loops designated as Mg1, Mg2, and Mg3 (Figure 3-8D). The midgut is composed of a single-layered epithelium resting on a continuous basement membrane (Figure 3-9A-C). Circular and longitudinal muscles lie just beyond the basement membrane and serve to contract the midgut. The microvilli of the midgut cells are distinct in each midgut region, and are surrounded either by a myelin-like membrane (Mg1-2) or glycocalyx that form a brush border (Mg3) (Figure 3-9D). The midgut is endodermal in origin, and because it lacks a cuticular intima, thrips have an extracellular membrane termed the perimicrovillar membrane (PM) that supposedly functions to compartmentalize digestion and/or aid in the absorption of essential amino acids. *Frankliniella fusca* have four Malpighian tubules at the base of the midgut which open into the digestive tract at the pyloric region where the midgut meets the ileum of the hindgut (Figure 3-10A). The two anterior tubules appear to be adhered for a short distance to the midgut and the two posterior tubules are connected to the ovarioles via small ligaments. The Malpighian tubules function together with the hindgut as excretory organs to remove waste materials from circulation within the hemolymph and to also reabsorb useful substances. Intracellular granules, spheres, and vacuoles are commonly found in the Malpighian tubules and serve as collection sites for nitrogenous waste and other excretory products (*e.g.*, uric acid as well as other organic and inorganic material) (Figure 3-10B-C). The hindgut (proctodaeum) constitutes the posterior part of the alimentary canal (Figure 3-8A). This rather simple tube extends from the pyloric valve to the anus and is supported posteriorly by suspensory muscles extending from the abdominal wall. The hindgut is derived from ectodermal tissue and lined by a thin layer of cuticular intima (Figure 3-11A). This cuticle functions to protect the underlying epithelial cells and is shed with each molt. The formation of primary urine in the Malpighian tubules is accompanied by a loss of useful substances and the hindgut
acts to selectively reabsorb those substances from the lumen back into the hemolymph. The hindgut also secretes additional waste components into the urine. Both the midgut lumen and the hindgut are associated with numerous bacteria (Figure 3-11B-D, Figure 5-1A-B).

The female reproductive system of *F. fusca* consists of the ovaries, calyx, two lateral oviducts, a median oviduct, a spermatheca, accessory glands, and a vagina or genital chamber. Females have one pair of panoistic ovaries consisting of two ovaries with four ovarioles each located underneath the alimentary tract. Each ovariole is divided into five zones: (1) the terminal filament; (2) the germarium; and (3-5) the vitellarium zones IIIa-c. The ovarioles merge posteriorly at the calyx and unify to a median oviduct which forms the vagina. The spermatheca is located just anterior to the vagina and serves as a storage organ for the sperm (Figure 3-12). The accessory gland is situated against the ovipositor and produces substances that attach eggs to the substrate during oviposition (Figure 3-12). The ovipositor is formed from a modification of the eighth and ninth abdominal segments, and females insert eggs into plant tissue with this structure (Figure 3-4). During oogenesis, the eggs fill nearly the entire abdominal cavity (Figure 3-13). Eggs are full of lipid droplets and proteins (yolk) that are transported from the fat body to the ovaries by the hemolymph (Figure 3-14). The developing oocytes are of different developmental stages and appear healthy with prominent and well-defined nuclei in the follicle cells (Figure 3-15). The follicle cells are tightly linked to surrounding the oocytes by ladder-like extensions (Figure 3-16).

The fat body of *F. fusca*, located as thin lobes immediately under the female thrips abdominal cuticle, is the major tissue for metabolism and nutrient storage (Figure 3-5). In this tissue, adipocytes store energy reserves as lipid droplets composed of triglycerides. There is also an abundance of glycogen in these cells. The fat body is the principal site for the synthesis of
vitellogenin, the egg yolk precursor protein. In addition, the fat body synthesizes antimicrobial peptides, hemocyte proteins, metabolites, storage proteins, lipophorins, and hormones. The fat bodies are heterogenous, appearing as smooth and regular masses with globules of fat droplets and glycogen deposits (Figure 3-17).

*Frankliniella fusca* possess hundreds of individual muscles (Figure 3-18). A typical insect muscle is composed of either bundles of or loosely aggregated muscle fibers made of myofibrils, each which contain actin, myosin, and contraction proteins in repeating sarcomere units (Figure 3-18B-C). Sarcoplasmic reticulum runs longitudinally on the surface of the muscle fibers and store calcium ions used in the contraction process (Figure 3-18C). The muscles also contain many large mitochondria (sarcosomes) and intracellular tracheoles to provide energy and oxygen for their use (Figure 3-18B-C).

*Thripinema fuscum*: Infective females (n=10). The average body length is 199 µm ± 17.80 µm (range of 175.3 µm – 221.0 µm); the average body width is 7.97 µm ± 1.02 µm (range of 6.65 µm – 9.8 µm). The female body is straight to slightly curved when heat-relaxed (Figure 3-19A). The cuticle is annulated with transverse striations averaging 0.55 µm and bearing lateral fields (=cuticular ridges) that extend the length of the body (Figure 3-19B–E, Figure 3-20). Each lateral field is 1.56 µm ± 0.26 µm wide and composed of two thick lateral lines measuring 0.25 µm ± 0.03 µm. Females have a distinct protrusible hollow stomatosylet composed of a stylet shaft and cone (etymology is “tyl”=knobbled; “enchos”=spear) (Figure 3-21). The mouth is moderately sclerotized and consists of a stoma (=pore) (0.20 µm × 0.65 µm) surrounded by four submedian lobes (Figure 3-22). Females have an excretory pore opening on the ventral side of the anterior region (Figure 3-23). The uterus is well-developed with a single prodelphic ovary.
Mature parasitic female (n=7). The average body length is 169.27 µm ± 47.31 µm (range of 143.5 µm – 219.8 µm); the average body width is 60.95 µm ± 17.52 µm (range of 36.82 µm – 91.48 µm). The female body is oval-shaped, often with part of uterus protruding from the vulva (Figure 3-24). A lateral field and excretory pore was not observed. Females lack a stylet and esophagus, and their body wall is modified with a hypodermal layer (Figure 3-25). Upon closer examination, the hypodermis is covered with microvilli and punctations (Figure 3-26). The ovary is long and convoluted and the uterus usually with one to two eggs.

Free-living male (n=1). The body length is 160.85 µm; the body width is 8.2 µm. Body is curved dorsally when heat-relaxed (Figure 3-27). The male cuticle is similar to the infective female and a lateral field is present. The stylet is indistinguishable (Figure 3-27). The posterior region possesses the following reproductive structures (Figure 3-28): caudal alae (=bursa) large with crenate margins (Figure 3-28B–D), paired spicules, and a thin well-sclerotized gubernaculum (Figure 3-28E–F).

Juveniles (n=13). The average body length is 124.31 µm ± 35.43 (range of 82.83 µm – 161.95 µm); the average body width is 9.88 µm ± 1.81 µm (range of 7.47 µm – 13.02 µm). Different stages of juveniles are found in *F. fusca*, ranging from small and wide for early-staged juveniles (J1–J2) to long and slender for late-staged juveniles (J2–J3) (Figure 3-29). The juveniles shed four cuticles (Figure 3-x) and the cuticle develops structure with each molt (Figure 3-30). Upon closer examination, the juvenile cuticle contains modifications that are likely for nutrient assimilation (Figure 3-31, Figure 3-32). The lip region and stylet develop in late-staged juveniles (Figure 3-33). Reproductive structures are not obvious at this stage.

Eggs (n=11). The average egg length is 34.03 ± 6.03 (range of 23.05 – 46.02); the average egg width is 16.64 ± 3.97 (range of 11.52 – 17.37). Eggs are oval-shaped (Figure 3-34)
and with bumps (Figure 3-35). The embryo is often observed through a transparent chorion (Figure 3-35).

**Life cycle:** A series of parasitized thrips sampled at intervals shows the generalized *in vivo* life cycle of a *T. fuscum* female (Figure 3-36). Immediately upon parasitization, the infectious female begins the dramatic transformation to the obese phenotype (Figure 3-36A). During this time (day one to day three post-parasitization), a mass deterioration of *F. fusca* body structure is apparent. The cuticle of *T. fuscum* degenerates and is replaced by a microvilliated layer interspersed with nodules and cuticular pits (Figure 3-25, Figure 3-26). The parasitic female is directly apposed to midgut cells where she likely sequesters nutrients for egg production (Figure 3-36A). The swollen female produces eggs within four days post-parasitization and eggs are continuously produced until death of either the host or parasite (Figure 3-36B). The eggs hatch into corpulent juveniles that morph to the characteristic vermiform juvenile shape. Fourth-stage juveniles emerge from the posterior end of male and female *T. fuscum* nine days post-parasitization, thus completing the life cycle. Typically, the female motherworm, eggs, and immature nematodes were found in the anterior abdominal regions of the thrips, whereas the fully mature juveniles were localized in a sac-like hindgut region (Figure 3-36C). Occasionally, the juveniles migrate to the thoracic and head tagmata (Figure 3-37). Diagrams of the complete *T. fuscum* life cycle can be seen in Figure 1-7 and Figure 3-38.

**Histopathology:** Externally, there are no detectable symptoms or morphological changes induced by parasitism even though the abdominal cavity of a *T. fuscum* parasitized female is filled with nematodes (Figure 3-39). However, light microscopy of thick sections demonstrated the significant impact of *T. fuscum* on the female thrips (Figure 3-40). Parasitization induces a displacement and invasion of the alimentary tract, an atrophy of the ovaries and fat body, an
accumulation of numerous electron-dense vesicles in hemocoel, and a degradation of muscle tissue.

Scanning electron microscopy of fractured parasitized *F. fusca* thrips revealed numerous juvenile nematodes oriented longitudinally in the abdominal hemocoel (Figure 3-41A-D). The densely-packed nematodes pressed against the midgut, forming indentions on the basal side of the basement membrane (Figure 3-41E-F). Transmission electron microscopy of thin sections from parasitized thrips revealed the parasitic *T. fuscum* female was always located proximal to the midgut with her microvilliated layer in direct contact with the basement membrane (Figure 3-42). The midgut cells appeared similar in size, shape, and organelle content to the cells of non-parasitized *F. fusca* (Figure 3-43). The midgut of parasitized *F. fusca* shows the presence of what appears to be a peritrophic matrix in the lumen (Figure 3-43D-F). This matrix was not visible in any of the healthy thrips, however, suggesting this exocellular secretion may instead be a sloughing of microvilli (Nation, personal communication). Late-staged juvenile nematodes move from the hemocoel and penetrate into the hindgut lumen where they form a mass aggregation (Figure 3-44A-C, F). Upon closer examination, this aggregate appears to be where mature males inseminate females prior to their exodus from the host (Figure 3-44D-E, Figure 3-46). Transmission electron microscopy of thin sections from parasitized *F. fusca* female shows the hindgut lumen full of late-staged juvenile nematodes (Figure 3-45). Examination of the alimentary tracts of the parasitized thrips revealed the absence of the microbiota associated with healthy thrips (Figure 3-45D-E, Figure 5-3). Lastly, the Malpighian tubules are full of secretory vesicles and uric acid crystals that probably serve as collection sites for *T. fuscum* excretory waste product filtered from the host hemolymph (Figure 3-47).
Gross dissection of parasitized thrips revealed the ovaries of parasitized *T. fuscum* were half the size of those in non-parasitized thrips (Figure 3-48). The reduced ovarioles were significantly displaced as a result of the numerous juvenile nematodes in the hemocoel (Figure 3-40). As a result, the zones of the oocytes were compressed against either juvenile nematodes or other host tissues (Figure 3-49). Transmission electron micrographs show a degradation of the oocytes with apoptotic bodies and nuclei of different sizes in the surrounding follicular epithelium (Figure 3-50).

In addition to the reproductive tissues, apoptotic bodies were present in fat deposits of parasitized *F. fusca* females. The atrophied fat bodies in parasitized thrips lacked lipid droplets but contained numerous dense vesicles filled with granular material (Figure 3-51A-B). There was a depletion of glycogen in many of the fat bodies as demonstrated by the space void of glycogen and organelles (Figure 3-51C-D). This depletion in glycogen coincided with an increased glycogen content inside juvenile nematodes (Figure 3-52). Parasitized *F. fusca* also had storages of glycogen in the abdominal muscle tissue (Figure 3-53A-C). Numerous lesions and ruptured mitochondria were also visible in the muscle tissue (Figure 3-53D-F). In many sections, progeny nematodes present in the hemocoel were partitioned away from insect tissues by an exocellular membrane that formed *Thripinema*-containing vacuoles void of insect cells (Figure 3-54, Figure 3-55).

**Discussion**

The internal morphology of non-parasitized *F. fusca* is similar to other thrips species (Ullman et al., 1989; De Bene et al., 1991; Dallai et al., 1991, 1996; Hunter and Ullman, 1992, 1994). In the following paragraph, I only discuss *F. fusca* morphological characters that differ from those previously described in the literature for *Frankliniella* sp. First, Dallai et al. (1991) reported that the anterior Malpighian tubules of *F. occidentalis* lie free in the hemocoel but that
the posterior tubules are briefly adhered to the hindgut wall before their separation into the hemocoel. This is not the case for *F. fusca*. The two anterior tubules appear to be adhered for a short distance to the midgut and the two posterior tubules are connected to the ovarioles via small ligaments. Second, Terebrantian thrips within the *Frankliniella* genus have a single accessory gland that is evident as a large apical bulb (Heming, 1970; Dallai et al., 1996; Kumm, 2002). The apical bulb of *F. fusca* is connected to the pyloric region via small ligaments. This is the first record of such an attachment in *Frankliniella* sp. and it can be assumed that either other species of *Frankliniella* do not have these ligaments or the fragile ligaments were broken during dissection.

Examination using light and electron microscopy provided insight into the life cycle of *T. fuscum*. The *T. fuscum* life cycle is typical to that of other Allantonematidae nematodes (Sharga, 1932; Lysaght, 1936, 1937; Nickle, 1963; Nickle and Wood, 1964; Ashraf and Berryman, 1970; Wilson and Cooley, 1972; Thong and Webster, 1975; Reddy et al., 1982; Tomalak et al., 1988; Chizhov et al., 1995; Green and Parrella, 1995; Teulon et al., 1997; Funderburk et al., 2002b; Poinar et al., 2004; Zeng et al., 2007). The free-living *T. fuscum* emerge from a parasitized male or female *F. fusca* host in the moist flower perianths of peanut where thrips aggregate to feed on pollen. The free-living juveniles can survive up to 48 h outside of a host under optimal conditions (data not shown). The infectious females remain in the flowers and, upon contact with a new host, enter the thrips through the intersegmental membranes of the abdomen or the coxal cavities of the leg. After the infectious female enters a thrips host, she undergoes a dramatic phenotypic transformation that is likely triggered by exogenous stimulation by host factors (Croll, 1970; Sukhdeo and Sukhdeo, 2004). The infectious *T. fuscum* female sheds her cuticle and transforms her epicuticle into a microvilliated layer with pits. The ultrastructure of
the integuments of Tylenchid parasitic female nematodes has been well documented (Riding, 1970; Poinar, 1972; Cliff and Baldwin, 1985; Subbotin et al., 1993, 1994, 1996). The female body surface contains a microvilliated layer with numerous ampullae and vacuoles on the outer hypodermal membrane. In *Skarbilovinema laumondi* (Tylenchida: Iotonchiidae), the microvilli of the hyperdermis are densely packed and form a “spongy layer”. The hypodermal processes (microvilli, ampullae, and vacuoles) are absorptive organs and the “spongy layer” aids in primary digestion (Subbotin et al. 1993). In my study, the parasitic *T. fuscum* female nestles against the host midgut and appears to directly absorb nutrients from the host midgut cells. This assimilation of nutrients provides the energy required for the substantial numbers of *T. fuscum* eggs produced. Reddy et al. (1982) reported up to 420 *Howardula aptini* eggs in a female *Megalurothrips* sp. host. Eggs laid by the parasitic *T. fuscum* female are released into the host hemocoel and hatch after two to three days. The eggs and juveniles are partitioned from the host hemolymph by a secreted layer or “surface coat”. This surface coat is an external, extra-cuticular labile layer that protects against host immunogens (Blaxter et al., 1992). Similar to the parasitic female, the juveniles likely sequester nutrients for development through their cuticle. In early-staged juveniles, there are cuticular pits on the surface which may aid in nutrient assimilation. Late-staged juveniles develop a definite cuticular layer and a functional stylet. Tomalak et al. (1988) reported juvenile *Sulphuretylenchus* spp. stylet feeding caused mass destruction to host beetle organs but *Neoparasitylenchus, Allantonema*, and *Contortylenchus* sp. caused only minor tissue damage to host beetles. I did not observe direct damage to host tissues by stylet feeding of juvenile nematodes in my study and hypothesize the stylet of juvenile *T. fuscum* functions to penetrate the host alimentary tract. Juvenile *T. fuscum* were never observed in the foregut or midgut lumen of parasitized individuals. Based on this observation, I suggest
late-staged juvenile nematodes (J3s) enter the alimentary tract through a pocket located at the pyloric valve where the proximal region of the Malpighian tubules meet the posterior midgut and anterior hindgut. Invasion of the nematodes into this junction has been documented by Serrao et al (2008) for nematode parasites of Hypocryphalus mangiferae (Coleoptera: Curculionidae). Male T. fuscum, who lack a functional stylet, would also be able to enter through this region.

After the late-staged male juveniles enter the alimentary tract, they migrate to the hindgut and aggregate to inseminate females prior to their exodus from the posterior region of the host. From an evolutionary perspective, it is preferential that the female nematodes mate inside the host rather than outside in the harsh environment where they are prone to desiccation and isolation. Often, more than one parasitic T. fuscum female parasitizes a thrips host which reduces consanguinity in the nematode populations.

Parasitism by T. fuscum causes substantial pathological changes to female F. fusca host tissues and organs. There was a significant displacement of host tissues that increased with nematode density. The tissues most significantly affected by T. fuscum parasitism were the fat body and reproductive tract.

First, fat bodies were reduced and replaced with large dense vacuoles. In addition, there was an obvious depletion of host glycogen in the fat bodies that was simultaneous with an observed glycogen increase in juvenile nematodes. Fat body is likely metabolized by the nematodes for energy, resulting in a significant reduction in this tissue. Parasite-induced depletion of host fat body tissue is a common outcome of parasitism (see Bailey and Gordon, 1973; Condon and Gordon, 1977; Schmidt and Platzer, 1980; Tomalak et al., 1984, 1990). The transfer of glycogen from F. fusca to T. fuscum suggests the parasite utilizes host glycogen as a carbohydrate/glucose source for body energy. Cheng and Snyder (1962) reported that cells
recently involved in glycogen digestion have aggregates of glycogen granules that form amorphous masses in the cytoplasm of host snail cells. As trematode parasitism progresses, host cell glycogen is depleted and an increase of glycogen within the parasites is observed. Decreased levels of glycogen or glycogenesis in fat body has also been reported for *Schistocerca gregaria* infected with *Mermis nigrescens* (Gordon and Webster, 1971), larval black flies *Prosimulium miuxtum, P. fuscum*, and *Simulium venustum* infected with *Neomesomermis flumenalis* (Condon and Gordon, 1977), mosquito *Culex pipiens* infected with *Romanomermis culicivorax* (Schmidt and Platzer, 1980), and *Lymantria dispar* infected with the microsporidium *Vairimorpha* sp. (Hoch et al., 2002). The dense vacuoles replacing contents of the fat body may be excretory *T. fuscum* waste product. Cheng and Snyder (1962) reported similar large clumps of granular material in snails infected with trematodes which “without doubt represent cercarial excreta”.

The second and most pronounced change induced by *Thripinema* in its *F. fusca* host is a reduction in size and shape of the reproductive organs (Lysaght, 1937; Green and Parella, 1995; Loomans et al., 1997; Arthurs and Heinz, 2003). *Thripinema fuscum*, operating as a “parasitic castrator”, causes a rapid sterilization of parasitized thrips (Sims et al., 2005). The time frame to induce sterility depends upon the stage is parasitized; adult female *F. fusca* parasitized as larvae do not produce any eggs whereas females parasitized as adults stop laying eggs within 2-3 days (Sims et al., 2005). The physiological mechanisms driving sterility are not well understood in this genus (but see Chapter 6). Lysaght (1937) suggested that *Thripinema* rendered female thrips sterile by either depriving thrips of protein required for normal development or by secreting a toxin that damages the reproductive organs. Hocking (1967) proposed that juveniles stopped oogenesis by directly feeding on the reproductive organs or associated tissue, a term otherwise
known as mechanical castration. Green and Parrella (1995) speculated that massive numbers of juvenile nematodes in the abdomen stimulate stretch receptors signaling the ovaries to halt oogenesis as if maximum egg capacity had been attained. Sims et al. (2005) concluded that the parasitic *T. fuscum* female is responsible for stopping oogenesis because sterility is induced before the production of juveniles in the host hemocoel. Numerous families within the Nematoda have been reported to induce partial or complete sterility in their insect host [e.g., Sphaerulariidae and *Musca autumnalis* (Treece and Miller, 1968), *Scolytus ventralis* (Ashraf and Berryman, 1970a,b), *Vespa simillima* (Sayama et al., 2007); Allantonematidae and *Scolytus* spp. (Oldham, 1930), *Dendroctonus pseudotsugae* (Thong and Webster, 1975), *Hypothenemus hampei* (Castillo et al., 2002); Neotylenchidae and *Sirex noctilio* (Bedding, 1972)], but few mechanisms for the observed sterility have been provided. Since pathogens and their host share the same resources, theory suggests that a parasitized host is forced to convert fecundity resources toward sustaining infection (longevity) or that the parasite consumes host resources that would otherwise be used for host reproduction (Beckage, 1985; Hurd, 1990; Bonds, 2006). Perlman and Jaenike (2003) cross-infected *Drosophila* spp. with different allopatric and sympatric *Howardula* spp. and concluded that sterility is host determined because an individual host response to infection (measured by degree of sterility) was the same regardless of the infecting nematode species. Roseler and Roseler (1973), who determined that the yolk proteins of parasitized queen *Bombus terrestris* are of a lower concentration than those of the non-parasitized conspecifics, suggested that the nematode causes injury to the yolk-producing corpora allata gland. *Tenebrio molitor* L. beetles parasitized by the rat tapeworm exhibit reduced fertility and fecundity and an increased lifespan (Hurd et al., 2001). The metacestodes produce a small effector peptide(s) that stimulates the transcription of vitellogenin mRNA but suppresses
translation resulting in decreased levels of vitellogenin in parasitized *Tenebrio* females (Webb and Hurd, 1999; Warr et al., 2006). In addition, components transiently present in the hemolymph retard the uptake of yolk proteins by the ovarian follicles (Major et al., 1997). Studies have suggested the unidentified effector(s) disrupt hormonal activity (Webb and Hurd, 1995). In addition to inhibiting vitellogenin synthesis, the tapeworms also induce resorption of the developing eggs that results from the induction of an apoptotic program in the fat body cells (Warr et al., 2005, 2006). Similarly, I found that parasitized *F. fusca* females had a significant reduction in fat body and apoptosis in the follicle cells surrounding the ovarioles. Based on these observations, I suggest *T. fuscum* nematodes deplete fat body (as described above) which reduces the synthesis of vitellogenin necessary for oocyte development and maintenance (see Chapter 6). Alternatively, the endosymbiont *Wolbachia* localized in host ovarioles may be dictating the reproductive biology of parasitized *F. fusca* (see Chapter 2).

The mechanism *T. fuscum* utilizes to escape detection by *F. fusca* probably involves deceiving the non-self recognition of the host immune system. Vetter et al. (1978) and others (Smith et al., 1981; Smith et al., 1983; Edwards et al., 1990; Politz and Philipp, 1992; Rockey et al., 1983; Fattah et al., 1986; Badley et al., 1987) reported nematodes can evade host immune factors by sloughing off their cuticle and any surface-bound antibodies. Mastore and Brivio (2008) added both insect parasitic *Steinernema feltiae* nematodes and their isolated cuticles to cultures of *Galleria mellonella* larval hemocytes and discovered that the parasite cuticle is essential for immunoevasion. Through a series of elaborate assays, they show that *S. feltiae* cuticular lipids bind host proteins and create a “coat” or disguise mechanism against host hemocyte recognition. The cuticular surface of *T. fuscum* is dynamic and responsive to the host as demonstrated by the transformation of the infective to the parasitic female. There were few
hemocytes observed in the hemolymph and phagocytosis, encapsulation, nodule formation, or melanization of nematodes in the host hemocoel was not observed. Scanning electron micrographs did show host factors attached to the cuticle of all *T. fuscum* stages. In many instances, the juvenile *T. fuscum* nematodes were partitioned from the host hemolymph by an exocellular membrane. These sequestration mechanisms potentially protect the nematodes from the up-regulated thrips cellular and humoral defense systems. However, further studies using cytochemical stains and/or cryo-sectioning and antibody or lectin probes are needed to determine the biochemical properties of the *T. fuscum* surface layer and its function in immune evasion (see Chapter 6).

In summary, parasitism by *T. fuscum* induces numerous morphological and behavioral alterations (*e.g.*, sterility, a reduction in feeding and vector competence) to its *F. fusca* host (see Sims et al., 2005 and Chapters 4 and 5). The ability of the naturally-occurring *T. fuscum* to induce these alterations without significantly impacting longevity or mortality make this nematode an important candidate for use in thrips integrated management strategies.
Table 3-1. Procedure for preparation of insect tissue for scanning (1–5) and transmission (1–6) electron microscopy.

<table>
<thead>
<tr>
<th>Step</th>
<th>Stage</th>
<th>Chemical</th>
<th>Time</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Fixation</td>
<td>2.5% gluteraldehyde</td>
<td>2 hrs @ RT or overnight @ 4°C</td>
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<tr>
<td>2</td>
<td>Buffer wash</td>
<td>0.1M Cacodylate buffer</td>
<td>3X @ 15 min. each</td>
</tr>
<tr>
<td>3</td>
<td>Post-fixation</td>
<td>1% osmium tetroxide</td>
<td>2 hrs @ RT or overnight @ 4°C</td>
</tr>
<tr>
<td>4</td>
<td>Water wash</td>
<td>ddH₂O</td>
<td>3X @ 15 min. each</td>
</tr>
<tr>
<td>5</td>
<td>Dehydration</td>
<td>10% ethanol</td>
<td>10 min</td>
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<tr>
<td></td>
<td></td>
<td>30% ethanol</td>
<td>10 min</td>
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<td>50% ethanol</td>
<td>10 min</td>
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<td></td>
<td></td>
<td>70% ethanol</td>
<td>10 min or overnight @ 4°C</td>
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<td>80% ethanol</td>
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<td>90% ethanol</td>
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<td>95% ethanol</td>
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<td>100% ethanol</td>
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<td></td>
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<td>100% acetone</td>
<td>15 min</td>
</tr>
<tr>
<td>6</td>
<td>Infiltration</td>
<td>25% resin/75% absolute acetone</td>
<td>4 hrs or overnight @ RT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50% resin/50% absolute acetone</td>
<td>4 hrs @ RT</td>
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<td></td>
<td></td>
<td>75% resin/25% absolute acetone</td>
<td>4 hrs @ RT</td>
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<td></td>
<td></td>
<td>100% resin</td>
<td>4 hrs or overnight @ RT</td>
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<tr>
<td></td>
<td></td>
<td>100% resin</td>
<td>6 hrs @ RT</td>
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Figure 3-1. The external dorsal view of the *Frankliniella fusca* female head tagma with antennae, compound eyes, and ocelli.
Figure 3-2. The mouthparts of a *Frankliniella fusca* female. (A) Ventral view showing the mouth cone (arrow); (B) A scanning electron micrograph of the tip of the mouth cone showing the numerous sensory pegs and stylet; (C) Maxillary stylet with labral pad and food canal.
Figure 3-3. The two wing morphs of *Frankliniella fusca*. (A–C) Scanning electron micrographs showing the wings of a macropterous female. The wings are fringed, with fine long setae; (D–F) Scanning electron micrographs showing the wing pads of a brachypterous female; (G) Macropterous male (left) and female (right) *F. fusca*; (H) Brachypterous male (left) and female (right) *F. fusca*. 
Figure 3-4. Scanning electron micrographs documenting the posterior opening of a *Frankliniella fusca* female. (A–E) Various views of the female’s genital opening and saw-like ovipositor (arrows) used to cut slits into plant tissue and deposit eggs; (F) The retracted ovipositor inside the abdomen of a *F. fusca* female (arrow).
Figure 3-5. A longitudinal thick section of a healthy *Frankliniella fusca* female. The female has an abundance of muscle in the thorax, a convoluted alimentary tract, numerous lobes of fat body, and a robust reproductive system with ovarioles containing lipid-filled eggs and an ovipositor.
Figure 3-6. The ovoid and tubular salivary glands of a *Frankliniella fusca* female. (A) A gross dissection documenting the ovoid salivary glands (osg) proximal to the esophagus in the thoracic region and tubular salivary glands (tsg) running parallel to the foregut; (B) A longitudinal thick section showing the ovoid salivary gland; (C–D) Transmission electron micrographs of the tubular salivary gland (tsg) with microvilli (mv) and numerous secretory vesicles (sv). tr=trachea, bm=basement membrane, cu=cuticle.
Figure 3-7. Transmission electron micrographs of *Tomato spotted wilt virus* in the ovoid salivary glands of a viruliferous *Frankliniella fusca* female. (A–B, D–E) Progressive enlargement of TSWV virions released from the salivary gland cell cytoplasm into the lumen; (C,F) Clusters of TSWV virions in the ovoid salivary gland cell of an infected female. Arrows point to a group of enveloped virions in the cytoplasm.
Figure 3-8. Digestive tract of the *Frankliniella fusca* female. (A) A longitudinal thick section showing the foregut, midgut, and hindgut; (B) A longitudinal section through the thorax showing the cuticle-lined foregut; (C) A longitudinal section through the abdomen showing the midgut coiled around the surrounding reproductive organs; (D) A longitudinal thick section through the abdomen showing the convoluted midgut and midgut lumen.
Figure 3-9. Transmission electron micrographs showing midgut cells of the *Frankliniella fusca* female. (A) Midgut epithelium with well-organized brush border (mv) extending into the gut lumen (lu) and regenerative cell. Note the well defined nucleus (n) and trachea (tr) apposing the basement membrane; (B) Midgut epithelial cell with invaginations (in) of the basement membrane (bm) and microvilli (mv) on the apical face forming a brush border; (C) Typical epithelial cell of a *F. fusca* midgut. Insects secrete secretory vesicles (sv) from the midgut cells to the lumen to help digest meals. Cells undertaking constitutive secretion are often characterized by suitable amounts of golgi bodies (g). These golgi complexes consist of parallel cistern and numerous small vesicles; (D) Transverse section through the microvilli of a *F. fusca* midgut with mitochondria (mt) and glycogen (gly) deposits.
Figure 3-10. Malpighian tubules of the *Frankliniella fusca* female. (A) Four Malpighian tubules extend from the digestive tract at the pyloric region between the posterior midgut and anterior hindgut. The two anterior tubules are adhered for a short distance to the midgut, and the two posterior tubules are connected to the ovarioles via small ligaments; (B) A transmission electron micrograph of a transverse section through a *F. fusca* Malpighian tubule. Microvilli (mv) form the brush border lining the lumen (lu) and the basal surface of the cells rest on a basement membrane (bm). The Malpighian tubules are surrounded by hemolymph (he); (C) Uric acid crystal (ua) within the cytoplasm of a Malpighian tubule. These crystalline spheres are formed from nitrogenous waste (inorganic salts, uric acid, urates, proteinaceous material, etc.) and are eventually released into the tubule lumen. Invaginations of the plasma membrane (in) allow for elasticity and an increase in surface area for exchanging ions and molecules. mt=mitochondria.
Figure 3-11. Transmission electron micrographs showing the hindgut of *Frankliniella fusca*. (A) The general rectal wall consists of a thin epithelial layer (ep) lined with cuticle (cu). Note the glycogen granules (gly) in the rectal epithelium; (B) The hindgut with vacuoles (v), muscle fibers (mf), and glycogen aggregates in the rectal epithelium. Note the cuticular intima (arrows) lining the hindgut lumen (lu); (C) The hindgut wedged between a developing oocyte (oo) and a rectal pad (rp). The hindgut lumen (lu) is filled with numerous bacteria (b) (see Chapter 5); (D) The rectal pad (rp) contains numerous pleomorphic mitochondria (mt), muscle fibers (mf), and nuclei (N) along the cuticle-lined border.
Figure 3-12. The genital region of a *Frankliniella fusca* female. The apical bulb of the accessory gland is connected to the pyloric region via small ligaments. The spermatheca is adhered closely to the vagina and ovipositor.
Figure 3-13. Longitudinal thick sections of the female *Frankliniella fusca* host documenting egg development. (A) A pre-vitellogenic female and a (B) fecund female. The reproductive structures are robust, with the ovarioles extending to the first abdominal segment and numerous eggs in the ovaries. Note how the well-developed ovaries flank the defined alimentary tract in healthy females.
Figure 3-14. Scanning electron micrographs of the abdominal cavity of a *Frankliniella fusca* female with egg. (A–B) A developing egg (arrows); (C) Interior structure of the developing egg (arrow); (D) Yolk protein (arrow) inside the egg.
Figure 3-15. The developing oocytes of a fecund *Frankliniella fusca* female. (A–C) The progressive magnification of the follicle cells and developing oocytes. The follicle cells are distinct with prominent and well-defined nuclei (n).

Figure 3-16. The progressive magnification of connections between a follicular cell and developing oocyte of a non-parasitized *Frankliniella fusca* female. (A) Developing oocyte; (B) Nucleus in the follicle cells and organelles within the cytoplasm of the oocyte; (C–D) The zipper-like or ladder-like connections between the two layers are strong and distinct.
Figure 3-17. The fat body of a *Frankliniella fusca* female. The fat body is composed of large lipid droplets (asterics) and small dense glycogen granules (arrowheads).

Figure 3-18. The musculature of *Frankliniella fusca*. (A) A thick section showing the various muscles in the thoracic cavity of *F. fusca*; (B) A longitudinal section of the thoracic muscle. The I band (I) contains thin actin filaments that extend from the Z line (Z) to the thick myosin filaments (A, A band). The arrow represents the lighter zone where the thick myosin filaments are in the absence of the actin filaments. Trachea (tr) typically indent the cell membrane of the muscle cells, thus minimizing the distance for oxygen diffusion. nf=nerve fibers; mt=mitochondria; (C) Thoracic muscles of *F. fusca*. An abundant supply of mitochondria (mt) is needed to fuel the muscle cells for flight. The circular fiber bundles (mf) are enclosed by the sarcolemma (sl) and the dark lines within the circular fibers are likely sarcoplasmic reticulum (sr). Z=z-lines.
Figure 3-19. Scanning electron micrographs documenting the external surface of the infective *Thripinema fuscum* female. (A) Ventral view of a *T. fuscum* female; (B–E) Side profile of a *T. fuscum* female with lateral lines extending anterior to posterior ends; (F) Anterior view of an infective *T. fuscum* female. Note the well-defined cuticular structure in all micrographs.

Figure 3-20. Transmission electron micrographs showing the cross-section of an infective *Thripinema fuscum* female with lateral lines (arrows). (A–B) Progressive magnification of the lateral lines of a developing nematode.
Figure 3-21. Differential interference contrast microscopic image (DIC) of an infective *Thripinema fuscum* female with anterior esophageal region.

Figure 3-22. The cephalic region of the infective female *Thripinema fuscum*. (A–B) Enface view showing square mouth plate and stylet lumen (black arrows); (C–D) Lateral view showing the labial region with lips (white arrows). Note the well-defined cuticular structure in the micrographs.
Figure 3-23. Scanning electron micrographs of the excretory pore of an infective *Thripinema fuscum* female. (A) The excretory pore is located on the ventral side beneath the esophageal region; (B) A higher magnification of the excretory pore and surrounding pore cuticle.
Figure 3-24. Differential interference contrast microscopic images (DIC) of the parasitic *Thripinema fuscum* female. (A, D) Dorsal view of a fecund *T. fuscum* female; (B) A fecund *T. fuscum* female with two eggs; (C) Two parasitic *T. fuscum* females; (E) A fecund *T. fuscum* female with egg emerging from the uterus; (F) A montage image of the parasitic *T. fuscum* female.
Figure 3-25. Scanning electron micrographs of the cuticular structure of the parasitic *Thripinema fuscum* female. (A) Dorsal view of a parasitic *T. fuscum* female with slightly everted uterus; (B–H) Progressive magnification of the cuticular structure of the *T. fuscum* female documenting the pit-like structures (F–G) and knob-like projections (E–H).
Figure 3-26. Transmission electron micrographs documenting the cuticular structure of the parasitic *Thripinema fuscum* female. (A) Cuticle of the parasitic female with microvilli (arrow); (B–D) Various micrographs showing the microvilliated cuticle; (E–F) Knob-like projections extending from the cuticle of the parasitic *T. fuscum*.
Figure 3-27. Differential interference contrast microscopic images (DIC) of the *Thripinema fuscum* male. (A-D) Arrows point to the copulatory structures of the bursa (arrow) and gubernaculum (arrowhead), a sclerotized structure used for guiding the spicule. Males are easily differentiated from females by their curved body structure.
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Figure 3-50. The progressive magnification of the connection between the follicular cells and developing oocyte in the *Thripinema fuscum* parasitized *Frankliniella fusca* female. (A) Nucleus in the follicle cell surrounding an oocyte; (B) The zipper-like or ladder-like connections between the oocyte and follicle cell are torn; (C) Apoptosis in the follicle cell of an oocyte characterized by a migration of heterochromatin to the edge of the nucleus and a poorly-defined nucleus.
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CHAPTER 4
THE IMPACT OF A PARASITIC NEMATODE *THRIPINEMA FUSCUM* ON THE FEEDING BEHAVIOR AND VECTOR COMPETENCE OF *FRANKLINIELLA FUSCA*

**Introduction**

The tobacco thrips, *Frankliniella fusca* (Hinds) (Thysanoptera: Thripidae), is a polyphagous insect pest that feeds on agriculturally important plants in North America (Lewis, 1997). It is one of at least seven thrips species capable of transmitting *Tomato spotted wilt virus* (TSWV, Bunyaviridae: Tospovirus) in crops such as groundnut, tobacco, tomato, pepper, as well as in numerous ornamentals, grasses, and weeds (Parrella et al., 2003; Whitfield et al., 2005). *Frankliniella fusca* populations contain both brachypterous and macropterous adults with the proportion of each wing form changing seasonally (Chamberlin et al., 1992). Ecologically, wing form plays a role in thrips dispersal (Chamberlin et al., 1992; Groves et al., 2003), yet no studies have investigated if wing form has a direct effect on TSWV replication and transmission.

Transmission of Tospoviruses by viruliferous adult thrips is the only significant form of inoculation during natural epidemics and the transmission rate is dictated primarily by the feeding behaviors exhibited by the respective vector (Culbreath et al., 2003; Ananthakrishnan and Annadurai, 2007). TSWV is transmitted in a propagative manner by thrips (Ullman et al., 1993; Wijkamp et al., 1993). Acquisition of TSWV occurs when first and early second instars feed on infective plant tissue, and transmission occurs via late instar and adult thrips that have acquired the virus as larvae (van de Wetering et al., 1996; Wijkamp et al., 1996b). Larvae and adults feed by piercing plant cells and sucking out the cell fluids, which produces a silvery scarring on the plant tissue (Hunter and Ullman, 1989). It has been difficult to resolve a consistent pattern in the pathological effects of tospovirus infection on thrips as there have been reports of negative (Stumpf and Kennedy, 2005), neutral (Wijkamp et al., 1996a), and positive (Maris et al., 2004; Stumpf and Kennedy, 2007) effects on thrips. Recent work has shown
genetic variation of both thrips populations and Tospovirus isolates, as well as the quality of infected host plant tissue, are important factors in pathogenicity (Stumpf and Kennedy, 2005; Stumpf and Kennedy, 2007). Because vector transmission varies based on both the quality and quantity of feeding of thrips (van de Wetering et al., 1998; van de Wetering et al., 1999), I hypothesize manipulation of feeding behaviors may provide an avenue for decreasing the spread of Tospoviruses in agroecosystems.

The insect parasitic nematode *Thripinema fuscum* Tipping and Nguyen (Tylenchida: Allantonematidae) renders female *F. fusca* sterile without any negative effects on their survival (Sims et al., 2005). Parasitism significantly reduces the longevity of male *F. fusca* and the effects of *T. fuscum* on male reproduction are unknown (Sims et al., 2005). Parasitism by *T. fuscum* is initiated when a free-living female enters the host through the intersegmental membranes of the coxal and/or abdominal cavities (Tipping et al., 1998). Once inside the host, the parasitic female produces eggs that hatch and develop through three juvenile stages before boring into the alimentary tract and emerging via the anus as the fourth-stage adult (Sharga, 1932). All stages of *F. fusca* are capable of being parasitized, with young adult females the most preferred (67%) and males least preferred (25%) in laboratory experiments (Sims et al., 2005). Parasitism of adult *F. fusca* has been reported to exceed 80% in field conditions (Funderburk et al., 2002). Parasitism of *F. fusca* larvae on groundnut averaged 49% and 28% in laboratory and field experiments, respectively (Sims et al., 2005). The lower rate of larval parasitism in field conditions is most likely due to differences in microhabitat between the larvae and parasitized females; larvae remain within the terminal buds of groundnut and parasitized adults typically aggregate in the flowers where they feed on pollen and where free-living *T. fuscum* are able to easily contact new hosts.
Recent observations suggest that parasitism by *Thripinema* spp. may suppress TSWV transmission by reducing host feeding rates (Sims et al., 2005). Related research has shown that the feeding by *Frankliniella occidentalis* (Pergande) is suppressed significantly by the nematode *Thripinema nicklewoodi* Siddiqi; however, the effects of parasitism on vector competence are unclear (Arthurs and Heinz, 2003; Lim and Van Driesche, 2004). Arthurs and Heinz (2003) reported that TSWV infection of *F. occidentalis* did not affect susceptibility to *T. nicklewoodi*, but fewer parasitized thrips became active transmitters and their per capita frequency of disease transmission was reduced by 50%. Alternatively, Lim and Van Driesche (2004) reported that parasitized *F. occidentalis* did not subsequently acquire Impatiens necrotic spot virus as readily as their non-parasitized counterparts but that rates of transmission remained the same between the two viruliferous groups.

The interaction between *F. fusca, T. fuscum*, groundnut, and TSWV serves as an ideal multi-trophic system to examine the impact(s) of a chronic disease on vector competence. The first objective of this chapter was to examine the effects of gender, wing form, virus infection, and nematode parasitism on the feeding behavior of *F. fusca*. The second objective was to determine what effects gender, age, and nematode parasitism have on TSWV transmission. From these experiments, I provide a framework for better understanding how the highly host specific *T. fuscum* parasite interfaces with plant viruses/insect vector associations.

**Materials and Methods**

**Maintenance of *F. fusca, TSWV inoculum, and T. fuscum***: Colonies of healthy and parasitized *F. fusca* were maintained as described by Sims et al. (2005). Leaves of groundnut (*Arachis hypogaea* L.) showing TSWV symptoms were collected in Alachua Co., Florida, and confirmed to be TSWV positive by double antibody sandwich enzyme-linked immunosorbent
assay (DAS-ELISA), a technique used to determine the presence or absence of TSWV by
detecting viral structural (nucleocapsid) proteins (SRA 39300; Agdia Inc., Elkhart, IN).

TSWV was maintained in groundnut by *F. fusca* transmission. For virus acquisition, first
instars were placed on infected tissue and allowed to feed for 24 hours. Larvae were then
transferred to 15-cm polypropylene containers with a 5-cm diameter ventilation hole covered
with fine mesh. The polypropylene containers were stored in a sealed plastic crisper lined with
moist paper towel and maintained at 27°C with a 14h light and 10h dark photoperiod. Fresh
tetrafoliate leaves were deposited into containers every day until adult emergence. Transmission
of the virus to ‘Florunner’ groundnut was achieved by allowing viruliferous adults to feed for 72
h on 3 to 6 wk old healthy plants enclosed in cages (12.7-cm diameter clear plastic cylinders with
6.35-cm diameter screen holes). Host plants were held at 25-30°C in a greenhouse. After an
incubation period of approximately 10-20 days following the end of the inoculation access
period, symptomatic plants were confirmed to be TSWV positive using DAS-ELISA.

**Effect of gender, wing form, virus infection, and nematode parasitism on
survivorship, feeding behavior and TSWV transmission:** Approximately 200 females were
confined for 48 hours in four cages containing TSWV infected groundnut for oviposition.
Newly eclosed first instars were allowed to feed on TSWV-infected leaves for a 48-hour
acquisition period to generate viruliferous thrips. After this 48-hour period, half of the larvae
from each cage were transferred in groups (n = 20) to 1.5-ml microcentrifuge tubes containing
two *T. fuscum*-parasitized adult female *F. fusca* excreting nematodes and a 1-cm diameter
groundnut leaf disc. Larvae were held with parasitized females for 72 h to achieve optimal
levels of parasitism. The remaining half of the larvae were transferred to tubes with two healthy
adult females and a 1-cm diameter groundnut leaf disc for 72 h to serve as non-parasitized
controls. Corresponding control thrips (i.e., uninfected by TSWV) were reared in the same manner, except for being given healthy groundnut leaves rather than TSWV infected groundnut leaves.

Individual larvae were then placed (after the 48 h virus acquisition period and 72 h parasitization access period) in tubes with a fresh groundnut leaf disc until adult emergence. After thrips emerged as adults, they were each transferred individually (n = 213) to a new tube that was provisioned with a fresh groundnut leaf disc (1 cm²) with the top (adaxial) surface placed upward every 24 h until death. Immediately after each 24 h feeding period, the upper surfaces of the discs were photographed for feeding injury (Auto-Montage Pro, Syncroscopy) and analyzed (SigmaScanPro 4.02, Jandel Corporation 1995) using a modified color defined protocol of Kerguelen and Hoddle (1999) that detected the amount of silvered areas caused by thrips feeding. Background noise was accounted for by subtracting the mean pixel count of unfed control leaf discs from the pixel count of leaf discs with feeding injury. Pixel counts were converted to area of feeding (in mm²). After measurements were taken, leaf discs were placed on sterile water in 24-well plates for 5 days at 25°C to amplify the virus titer. Leaf discs were then stored at -70°C until analysis by DAS-ELISA. Leaf discs were recorded as positive if their ELISA (optical density) value was greater than the determined threshold value for the plate (mean of control leaf disc readings + 3[SD]).

At death, thrips were dissected and the gender, wing form, and presence of T. fuscum were recorded. Dissected thrips were then stored individually in tubes with 150 µl of PBS-PVP buffer at -80°C until further analysis could be conducted using antigen coated plate (ACP)-Indirect ELISA to confirm presence of viral replication in adults. The monoclonal antibody probes (Ascites cell lines 1C1A7 lot# A16714 and ascites cell line 6B1C1 lot# A16779; Agdia Inc.
Elkhart, IN) used in these assays detect a non-structural protein encoded by the small RNA segment of TSWV, thus differentiating thrips in which the virus is actively replicating and are capable vectors from those that have merely ingested the virus by feeding on infected plant tissue (Bandla et al., 1994).

**Statistical analyses:** To determine survivorship rates, thrips were classified according to gender and *T. fuscum* parasitism, giving four treatment groups for non-viruliferous thrips (non-parasitized females, parasitized females, non-parasitized males, parasitized males) (n = 144). However, because only two parasitized, viruliferous males, which died within one day, were obtained, only three treatment groups were available for viruliferous thrips (non-parasitized females, parasitized females, non-parasitized males) (n = 67). This classification enabled us to make similar analyses for viruliferous and non-viruliferous thrips.

Survival distribution curves according to gender, virus infection and nematode parasitism were generated using Kaplan-Meier estimates (Proc LIFETEST, SAS 2004), and the Cox Proportional hazards model (Proc TPHREG, SAS 2004) was used to determine how survival rates of adults differed among the four treatment groups of non-viruliferous thrips (non-parasitized females, parasitized females, non-parasitized males, parasitized males) and among the three treatment groups of viruliferous thrips (non-parasitized females, parasitized females, non-parasitized males). Comparisons were made to examine the impact of parasitism status and gender on host longevity.

For the feeding and transmission analyses, a subset of 10 individuals per treatment (5 of each wing form) that survived 10 d or more were randomly selected (RANDBETWEEN function, Excel 2000). It was not possible to determine the parasitism and viral status of thrips until after all experiments had been conducted, therefore I was unable to have an equal number
of thrips for all categories. The effects of independent variables on the amount of feeding were assessed using a mixed model repeated measures ANOVA with data normalized via a log (y+1) transformation before analysis (Proc MIXED, SAS 2004). The daily amount of feeding for each individual thrips was the repeated measure. Because of the potential serial correlations in feeding within each thrips, I used a first order autoregressive covariance structure in the models. Due to the lack of viruliferous males parasitized by *T. fuscum*, initial analyses were run separately for females and males to determine the effects of wing form, virus infection and parasitism on feeding injury. These initial models showed that wing form and virus infection did not affect feeding by males and females and were therefore taken out of the analyses. Further analyses (slice option within Proc MIXED, SAS 2004) were conducted to determine the effects of gender and nematode parasitism, and their interaction on feeding over time.

To observe transmission over time, mean cumulative distribution plots were generated for each treatment to (Proc RELIABILITY, SAS 2004). To determine if overall rates of transmission varied (*i.e.*, number of days with transmission/total days of adulthood), comparisons of transmission frequencies among the three groups of viruliferous thrips (non-parasitized females, parasitized females, non-parasitized males) were conducted using a generalized linear mixed model ANOVA (Proc GENMOD, SAS 2006). In this case, transmission was a binary response variable because each observed leaf disc was either infected or not infected. The generalized linear mixed model accounted for the response variable being expressed as the proportion of leaf discs infected relative to the number of leaf discs observed for each individual (Madden et al., 2002). Pairwise comparisons among the treatment groups were made using the LSMeans option.
To determine if the likelihood of transmission changed over the course of a thrips’ lifetime, and if this was related to their gender or parasitism status, I analyzed the gap times from one transmission event to the subsequent one for the three types of viruliferous thrips (non-parasitized females, parasitized females, non-parasitized males) [(Johnston and So, 2003; Nelson, 2003) Proc TPHREG, SAS 2004]. The robust sandwich estimate for the covariance was used to account for potential correlations in gap times within individuals.

To determine if the amount of feeding each day affected the likelihood of a thrips transmitting TSWV on that day, I analyzed if the probability of transmission increased with the amount of feeding for each of the three viruliferous thrips states. For these analyses, I used a logistic analysis, with a repeated effect being the 10 daily observations for each thrips (Proc GLIMMIX, SAS 2006). Amount of feeding was log transformed before analysis. Analyses were conducted separately for the three groups of viruliferous thrips because of differences in the amount of feeding among the groups (see results below).

**Results**

**Survivorship:** The longevity of non-viruliferous thrips was first compared to determine if T. fuscum parasitism affected the longevity of females and males (Figure 4-1A). Comparisons of the longevity of parasitized and non-parasitized thrips of each gender showed that females did not differ in their longevity ($\chi^2_1=0.08, P = 0.78$), but parasitism led to a significant reduction in longevity among males ($\chi^2_1= 16.77, P < 0.0001$) (Table 4-1). The proportional hazard ratios were similar between each comparison except for parasitized males; there was a 78% chance that a parasitized male would die before a non-parasitized male. The survival distribution graphs showed no difference in longevity between non-viruliferous and viruliferous F. fuscum cohorts, however, the onset of mortality occurred earlier for viruliferous thrips than for non-viruliferous
cohorts (Figure 4-1). Longevity did not differ between viruliferous non-parasitized ($\chi^2_1 = 0.55, P = 0.46$) and parasitized ($\chi^2_1 = 0.67, P = 0.41$) female thrips (Figure 4-1B, Table 1).

Feeding behavior: The feeding rates for female *F. fusca* were much higher than for males ($F_{1,60} = 119.34, P < 0.0001$) (Figure 4-2). There was a significant interaction between thrips gender and parasitism status ($F_{1,60} = 16.24, P = 0.0002$) on the feeding behavior of *F. fusca* adults indicating that the effect of parasitism on feeding differed between females and males. Parasitized females fed significantly less than non-parasitized females ($F_{1,38} = 54.82, P < 0.0001$), however there was no significant difference in the feeding rates of parasitized and non-parasitized males ($F_{1,22} = 1.25, P = 0.28$). There were no significant differences in the mean feeding rates within genders between brachypterous and macropterous individuals or within genders between viruliferous and non-viruliferous individuals (Table 4-2).

Because the 3-way interaction between gender, parasitism and time ($F_{9,540} = 0.84, P = 0.58$) was not significant and the resulting model with two-way interactions had a lower Akaike’s Information Criterion, the 3-way interaction was deleted from the analysis. The lack of an interaction suggested that differences in feeding between parasitized and non-parasitized thrips within each gender were consistent over time. There was not a significant gender by time interaction ($F_{9,540} = 1.33, P = 0.22$), indicating that females and males showed similar day-to-day variation in feeding (Figure 4-2).

Transmission rates: There were significant differences in transmission rates of TSWV among the three different groups of viruliferous thrips ($\chi^2_2 = 46.49, P < 0.0001$). Non-parasitized females transmitted more than non-parasitized males ($\chi^2_1 = 19.54, P < 0.001$). Parasitism significantly reduced virus transmission by females ($\chi^2_1 = 41.48, P < 0.0001$). There were no significant differences in transmission rates of parasitized females and non-parasitized males ($\chi^2_1$
= 1.52, P = 0.22). There were significant differences in the gap times between transmissions among the three groups of viruliferous thrips ($\chi^2_2 = 13.78, P = 0.001$), with viruliferous non-parasitized females having a 67% and 74% chance of transmitting before a viruliferous parasitized female and a viruliferous non-parasitized male, respectively. There were also significant differences in the time during the thrips life that transmission occurred ($\chi^2_1 = 41.34, P < 0.0001$). The mean cumulative distribution plots showed for all treatments that the frequency of transmissions decreased with age; older thrips did not transmit as efficiently as younger thrips (Figure 4-3, Table 4-3). The hazard ratio for the overall comparison was ~0.90, so the likelihood of a thrips transmitting TSWV decreased about 10% for each additional day of adulthood. This decline in transmission with age was consistent across categories of thrips ($\chi^2_2 = 0.76, P = 0.68$), reinforcing the idea that parasitism reduced transmission throughout adulthood. Non-parasitized females transmitted at a greater rate than both parasitized females and non-parasitized males, and these differences were consistent throughout the thrips lifetime (Figure 4-3).

The likelihood of transmission increasing with the amount of feeding per day differed according to parasitism status of the thrips. For non-parasitized females and males, the likelihood of transmission did not increase with their amount of feeding each day. The regression slopes were not significantly greater than 0 for non-parasitized females ($t_{89} = 1.31, P = 0.10$) or for non-parasitized males ($t_{89} = 0.94, P = 0.18$). In contrast, the likelihood of parasitized females transmitting on a particular day increased with their amount of feeding ($t_{80} = 1.87, P = 0.03$).

**Discussion**

The comprehensive nature of this research allowed for comparisons in feeding and transmission rates to be made for both male and female thrips and provided insight into how *T.*
fuscum may be modulating the physiology of its obligate F. fusca host. Feeding rates of female F. fusca were reduced nearly 65% by T. fuscum parasitism, a conclusion that was consistent with results from previous studies on T. nicklewoodi parasitizing F. occidentalis (Lim and Van Driesche, 2004; Arthurs and Heinz, 2003). Arthurs and Heinz (2003) proposed that parasitized female thrips fed less because developing T. nicklewoodi juveniles distended the abdomen and triggered stretch receptors of F. occidentalis. However, feeding rates of parasitized females were reduced immediately upon adult emergence and juveniles were not observed in the host hemocoel until much later. Furthermore, parasitism by T. fuscum did not appear to affect the feeding rates of male thrips in our study. Although male F. fusca produced fewer T. fuscum, they are significantly smaller than females, and likely, fewer T. fuscum would distend their abdomen in an amount proportionate to parasitized females (Sims et al., 2005; Reitz et al., 2006). Because male thrips feeding behavior was not affected by T. fuscum parasitism, it is also unlikely that T. fuscum is disrupting host neurological systems or that stomach lesions resulting from the continual migration of T. fuscum progeny into and out of the alimentary tract reduce the host’s ability to ingest food. A much more likely hypothesis is that F. fusca may be diverting nutrients from egg production towards sustaining Thripinema development (Hurd, 1990). Previous studies show that adult diet affects egg production of thrips and more energy (i.e., food intake) is likely needed for thrips oogenesis than is needed for sustaining T. fuscum infection (Teulon and Penman, 1991).

Despite the fact that T. fuscum reduced the amount of feeding by female F. fusca, parasitism did not decrease their longevity. Still T. fuscum parasitism had pathological effects on males, as parasitized males had lower survivorship than non-parasitized males despite feeding at similar levels. Lim et al. (2001) suggested that because healthy male thrips feed less than
females, their limited nutrient reserves are depleted quicker when parasitized by *Thripinema* thus reducing their longevity.

*Tomato spotted wilt virus* infection does not appear to have a significant effect on *F. fusca* longevity, which supports the findings of Wijkamp et al. (1996a) and Arthurs and Heinz (2003) for *F. occidentalis*. These results suggest that viruliferous adult *F. fusca* have a delayed onset of mortality, and this phenomenon may be an adaptive strategy of TSWV to enhance transmission by its host. The efficiency with which thrips transmit TSWV decreased with age. van de Wetering et al. (1999) suggested that TSWV transmission is a function of food ingestion; a higher consumption rate is associated with more viral particles being egested into plant tissue. Because feeding rates were consistent over the course of the thrips lifetime but transmission declined in our study, I hypothesize that viral titer may be reduced in older thrips because of senescence of salivary glands and/or degradation of viral particles.

In this study, non-parasitized females transmitted TSWV more efficiently than non-parasitized males. Sakurai et al. (1998), van de Wetering et al. (1998), and van de Wetering et al. (1999) reported that male *F. occidentalis* transmit with a higher efficiency than females because of differences in feeding behavior. Females tend to feed more frequently and for longer intervals, which irreversibly destroys cell contents and can prevent viral replication within the target plant. In contrast, males feed with a higher frequency of shallow probing and induce only minor cell damage so the cells are better able to support viral infection. The difference in transmission efficiencies between genders in this study and those mentioned may be due to differences in thrips species and host plants used, as both Sakurai et al. (1998) and van de Wetering et al. (1998, 1999) tested the effects of TSWV transmission by *F. occidentalis* on *Datura stramonium* (L).
*Thripinema fuscum* parasitism reduced TSWV transmission of adult female *F. fusca* by approximately 50%. Non-parasitized females fed more and had much higher transmission rates than parasitized females. This difference in transmission remained relatively constant throughout adulthood, which emphasizes the permanent impact of parasitism on thrips vector competence. Prior virus transmission data (Sakimura, 1963) has shown transmission of TSWV by viruliferous *F. fusca* adults to be sporadic. Transmissions for both non-parasitized and parasitized females in our study were sporadic and the gap times between transmissions were greater for parasitized females. I initially suspected vector capabilities would be reduced because *T. fuscum* lowers host feeding, and by doing this, reduces the viral titers delivered into plant tissue. This trend was observed when comparing the feeding and transmission rates for males and females - females fed more and transmitted at a higher rate than males. However, non-parasitized males fed less than parasitized females but transmitted TSWV at similar rates, suggesting other mechanisms may be operating to reduce transmission by parasitized females. Results from the logistic analysis may indicate that parasitism not only reduces feeding (which indirectly affects transmission), but also that parasitism has some type of direct effect on virus replication. It may be that *T. fuscum* is sequestering important nutrients from the host that are required for successful development, and by doing so, alters the physiology of salivary gland cells and their ability to replicate virus. Alternatively, it has been shown that activation of the immune system of *F. occidentalis* by TSWV infection induces the upregulation of antimicrobial and other immune system-related proteins (Medeiros et al., 2004). It is possible that both parasitism and TSWV infection may be inducing a synergistic immune response in the thrips that is detrimental to TSWV development.
Allantonematid nematodes induce various behavioral and physiological changes in their insect hosts (Hurd, 1993). By sterilizing female *F. fusca*, *T. fuscum* aids in reducing secondary spread of TSWV in field conditions (Sims et al., 2005). By reducing feeding, *T. fuscum* also aids in reducing primary spread of TSWV. Experiments in Chapter 5 were designed to determine whether *T. fuscum* reduces primary spread by inducing a direct effect on the titer of plant pathogens in the *F. fusca* vector. Understanding how these alterations influence vector competence may one day provide targets for suppressing disease spread.
Table 4-1. Mean longevity (± SE) of adult *Frankliniella fusca*.

<table>
<thead>
<tr>
<th>Parasitism status</th>
<th>Viruliferous(^1)</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not parasitized</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>15.15 ± 0.74 (52)</td>
<td>13.17 ± 1.06 (30)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>15.21 ± 0.72 (39)</td>
<td>15.4 ± 1.4 (10)</td>
</tr>
<tr>
<td></td>
<td>Parasitized</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>14.74 ± 1.03 (46)</td>
<td>8.25 ± 0.72 (16)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>17.72 ± 1.51 (18)</td>
<td>1.0 ± 0 (2)</td>
</tr>
</tbody>
</table>

\(^1\)Thrips were categorized as viruliferous if they tested positive for the NSs protein of TSWV by ACP-Indirect ELISA. Numbers in parentheses represent the total numbers of thrips per cohort.

Table 4-2. Mean total area of feeding (± SE) on leaf discs (mm\(^2\)) fed on by *Frankliniella fusca* individuals for the initial 10 days of adulthood.

<table>
<thead>
<tr>
<th>Parasitism status</th>
<th>Viruliferous(^1)</th>
<th>Wing form</th>
<th>Total feeding over 10 days (mm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not parasitized</td>
<td>Brachypterous</td>
<td>Females</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>3.60 ± 0.76 (5)</td>
<td>0.72 ± 0.13 (5)</td>
</tr>
<tr>
<td></td>
<td>Macropertorous</td>
<td>4.37 ± 0.80 (5)</td>
<td>0.35 ± 0.10 (5)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>3.07 ± 0.38 (5)</td>
<td>0.30 ± 0.05 (5)</td>
</tr>
<tr>
<td></td>
<td>Brachypterous</td>
<td>3.80 ± 0.68 (5)</td>
<td>0.51 ± 0.13 (4)</td>
</tr>
<tr>
<td></td>
<td>Macropertorous</td>
<td>1.32 ± 0.21 (5)</td>
<td>0.31 ± 0.08 (3)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>1.15 ± 0.16 (5)</td>
<td>0.34 ± 0.13 (2)</td>
</tr>
<tr>
<td></td>
<td>Brachypterous</td>
<td>1.33 ± 0.25 (5)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Macropertorous</td>
<td>1.24 ± 0.21 (5)</td>
<td>-</td>
</tr>
</tbody>
</table>

Sample sizes are given between parentheses (total n = 64).

\(^1\)Thrips were categorized as viruliferous if they tested positive for the NSs protein of TSWV by ACP-Indirect ELISA.

\(^2\)No parasitized viruliferous males survived beyond 1 day.
Table 4-3. Proportion of viruliferous *Frankliniella fusca* cohorts transmitting TSWV each day.

<table>
<thead>
<tr>
<th>Day</th>
<th>Non-parasitized females</th>
<th>Parasitized females</th>
<th>Non-parasitized males</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5 (10)</td>
<td>0 (10)</td>
<td>0.22 (9)</td>
</tr>
<tr>
<td>2</td>
<td>0.7 (10)</td>
<td>0.3 (10)</td>
<td>0.56 (9)</td>
</tr>
<tr>
<td>3</td>
<td>0.8 (10)</td>
<td>0.4 (10)</td>
<td>0.44 (9)</td>
</tr>
<tr>
<td>4</td>
<td>0.9 (10)</td>
<td>0.3 (10)</td>
<td>0.33 (9)</td>
</tr>
<tr>
<td>5</td>
<td>0.7 (10)</td>
<td>0.3 (10)</td>
<td>0.33 (9)</td>
</tr>
<tr>
<td>6</td>
<td>0.6 (10)</td>
<td>0.1 (10)</td>
<td>0.33 (9)</td>
</tr>
<tr>
<td>7</td>
<td>0.5 (10)</td>
<td>0.4 (10)</td>
<td>0.11 (9)</td>
</tr>
<tr>
<td>8</td>
<td>0.8 (10)</td>
<td>0.4 (10)</td>
<td>0.67 (9)</td>
</tr>
<tr>
<td>9</td>
<td>0.8 (10)</td>
<td>0.5 (10)</td>
<td>0.33 (9)</td>
</tr>
<tr>
<td>10</td>
<td>0.7 (10)</td>
<td>0.3 (10)</td>
<td>0.78 (9)</td>
</tr>
<tr>
<td>11</td>
<td>0.86 (8)</td>
<td>0.67 (9)</td>
<td>0.33 (6)</td>
</tr>
<tr>
<td>12</td>
<td>0.5 (8)</td>
<td>0.11 (9)</td>
<td>0.17 (6)</td>
</tr>
<tr>
<td>13</td>
<td>0.5 (6)</td>
<td>0.25 (8)</td>
<td>0.33 (6)</td>
</tr>
<tr>
<td>14</td>
<td>0.33 (6)</td>
<td>0.29 (7)</td>
<td>0.33 (6)</td>
</tr>
<tr>
<td>15</td>
<td>0.67 (6)</td>
<td>0.14 (7)</td>
<td>0.17 (6)</td>
</tr>
<tr>
<td>16</td>
<td>0.5 (4)</td>
<td>0.17 (6)</td>
<td>0.17 (6)</td>
</tr>
<tr>
<td>17</td>
<td>0 (4)</td>
<td>0.17 (6)</td>
<td>0.17 (6)</td>
</tr>
<tr>
<td>18</td>
<td>0 (3)</td>
<td>0 (5)</td>
<td>0.25 (4)</td>
</tr>
<tr>
<td>19</td>
<td>0.33 (3)</td>
<td>0 (4)</td>
<td>0 (2)</td>
</tr>
<tr>
<td>20</td>
<td>0.33 (3)</td>
<td>0 (4)</td>
<td>0 (2)</td>
</tr>
<tr>
<td>21</td>
<td>0 (2)</td>
<td>0 (4)</td>
<td>0 (1)</td>
</tr>
<tr>
<td>22</td>
<td>0.5 (2)</td>
<td>0 (3)</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>0 (2)</td>
<td>0 (3)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>0 (1)</td>
<td>0 (1)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0 (1)</td>
<td>0 (1)</td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>0 (1)</td>
<td>0 (1)</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>0 (1)</td>
<td>0 (1)</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses represents the number of thrips per cohort alive on that day.
Figure 4-1. Proportion of (A) non-viruliferous (n = 144) and (B) viruliferous (n = 69) *Frankliniella fusca* individuals surviving throughout adulthood.
Figure 4-2. Daily damaged area (mean ± SE) on leaf discs (in mm$^2$) fed on by *Frankliniella fusca* individuals for the initial 10 days of adulthood (n = 64).
Figure 4-3. Mean cumulative frequency (MCF) of TSWV transmission to individual leaf discs over the lifetime of adult *Frankliniella fusca*. The flattening of all three curves indicates that the frequency of transmission decreases with thrips age.
CHAPTER 5
THE MULTITROPHIC INTERACTIONS BETWEEN THE PARASITIC NEMATODE
THRIPINEMA FUSCUM, PLANT PATHOGENS PANTOEA ANANATIS AND TOMATO
SPOTTED WILT VIRUS, AND THE INSECT VECTOR FRANKLINIELLA FUSCA

Introduction

Frankliniella fusca is an important vector of viral and bacterial plant pathogens (see Chapter 1). The obligate parasite T. fuscum significantly reduces the vectoring capacity of F. fusca to transmit TSWV (see Chapter 4). Most, but not all, of the reduction in TSWV transmission to groundnut could be explained by a decrease in feeding rates of parasitized F. fusca. There were no significant differences in transmission rates of parasitized females and non-parasitized males, but males fed significantly less than parasitized F. fusca females. In addition to altering host feeding behavior, parasitism by T. fuscum potentially could induce a host response detrimental to plant pathogens in the insect vector.

Information on the impact of intra- and extracellular parasites/pathogens on the competence of insect vectors to transmit disease agents is scarce. One system that has demonstrated an impact on vector competence involves microsporidian intracellular parasites of malaria vectors (Aedes and Anopheles spp.) (Becnel, 1993). Microsporidian infection increases mortality, reduces longevity, and decreases the reproductive capacity of mosquito vectors (Undeen and Alger, 1975; Haq et al., 1981; Kelly et al., 1981). In addition to reducing population densities, infection also interferes with the plasmodial development in both Anopheles and Aedes vectors. Mosquitoes infected with microsporidia are less susceptible to Plasmodium infection (Bano, 1958; Hulls, 1971; Bargielowski and Koella, 2009). Bano (1958) found plasmodial development was inhibited as reflected by a decrease in both oocyst size and in number of ookinetes infecting the midgut. He speculated microsporidia may (1) sequester nutrients required for proper development of Plasmodium, (2) alter midgut cells and preventing
penetration by *Plasmodium* oocysts, or (3) cause the Malpighian tubules to releases toxic substances that interfere with *Plasmodium* development. Additionally, Hulls (1971) observed degenerated oocysts in the midgut lumen of *Anopheles* infected with microsporidia, a significant decrease in sporozoites in the salivary glands, and a reduction in sporozoite viability. Recent work suggests microsporidia infection “primes” the host immune system, thus impeding the development of *Plasmodium* parasites. For example, Bargielowski and Koella (2009) injected Sephadex beads into microsporidia-infected *Anopheles* and found an increased melanization response and reduced number of *Plasmodium* oocysts.

In addition to the limited research on microsporidia, both the gut microflora and endosymbionts of insects have been reported to impact vector competence. Ingestion of a blood-meal has been shown to stimulate bacterial replication in the midgut of mosquitoes (De Maio et al., 1996). Pumpuni et al. (1993) orally challenged adult *Anopheles* with a gametocyte culture spiked with bacterial preparations. Examination of these mosquitoes revealed a reduction in the number oocysts associated with the midgut tissue. The observed reduction in oocyst density was positively correlated to bacterial concentration. The authors speculated that bacteria may be non-specifically binding to the parasite or midgut epithelium, thus preventing entry of *Plasmodium* ookinetes into midgut cells. Additionally, Dong et al. (2009) demonstrated bacteria within *Anopheles* midgut inhibit infection of *Plasmodium*; removal gut bacteria through antibiotic treatment resulted in a near doubling of the oocysts in the midgut. Dong *et al.* (2009) fed mosquitoes a *Plasmodium*-infected blood meal containing either live or heat-killed bacteria and found fewer oocysts developed in the midguts. Interestingly, injection of bacteria into the hemocoel prior to challenge with *Plasmodium*-infected blood significantly reduced the number of oocysts in the midgut. These results suggested an indirect effect of the bacteria on plasmodial
development. Utilizing a microarray-based genome-wide gene expression strategy, Dong et al. (2009) like other investigators (Dimopoulos et al., 1996, 1997; Barillas-Mury et al., 1996; Hernandez-Martinez et al., 2002; Hillyer et al., 2003; Aguilar et al. 2005) found bacterial challenge to *Anopheles* upregulates the innate defense resulting in the production of immune genes (e.g., cercopins, defensins, gambicin, serine proteases, and pattern recognition receptors). These antimicrobial components disrupt the gut microflora that serve as a defensive barrier against *Plasmodium* development. The inhibition of parasite development by gut bacteria has been reported in other insect vectors including triatomines, tsetse flies, and sand flies (Schlein et al., 1985; Welburn and Maudlin, 1999; Azambuja et al., 2004).

In addition to manipulating reproduction (see Chapter 2), endosymbiotic bacteria such as *Wolbachia* protect insects from viral infection. Hedges et al. (2008) compared survival rates of *Drosophila melanogaster* infected with a range of pathogenic RNA viruses in the presence or absence of Wolbachia, and discovered a delay in virus accumulation and mortality in *Wolbachia*-infected flies. Teixeira et al. (2008) reported a laboratory *D. melanogaster* strain treated with tetracycline had higher viral titers of the same RNA viruses. The authors speculate *Wolbachia*-induced host resistance to RNA viruses may be caused by either a cell-autonomous or systemic effect. Autonomously, *Wolbachia* could compete for resources in the cell cytoplasm, reduce cell metabolism, and/or actively interfere with viral replication in the cell. Systemically, *Wolbachia* may pre-activate the host immune system allowing for a faster response upon viral infection. Recently, *Wolbachia* endosymbionts have been shown to modulate infection with Dengue, Chikungunya, Plasmodium and *Brugia* nematodes in the *Aedes* vector (Kambris et al., 2009; Moreira et al., 2009; Mousson et al., 2010). *Wolbachia* infection likely activates the innate immune system and renders the host more resistant to pathogen invasion and replication. The
tradeoff for constant immune upregulation is a shortened lifespan (Libert et al., 2007). The life shortening phenotype also reduces disease transmission by reducing the number of older (i.e., those that have surpassed the pathogen’s extrinsic incubation period) individuals within a vector population (Cook et al., 2008).

To my knowledge, the only work examining the impact of a parasite on competence of insect vectors in a plant system has been conducted on *Thripinema* spp. (see Chapter 1). Research findings concurred that the *Thripinema*-induced reductions in host feeding is a significant factor for the decrease in *Tospovirus* transmission rates (Arthurs and Heinz, 2003; Lim et al., 2004; Sims et al., 2008; see Chapter 4). The goal of this chapter was to observe if parasitism by *T. fuscum* impacts the titer of the plant pathogenic TSWV and *P. ananatis* in the *F. fusca* vector. Specifically, the first objective was to survey the cultivable bacterial fauna associated with field and laboratory *F. fusca* populations, and investigate the impact of *T. fuscum* parasitism on the capacity of *F. fusca* to harbor the plant bacterial pathogen *P. ananatis*. The second objective was to quantify and compare TSWV titers and TSWV transmission rates between non-parasitized and parasitized adult female *F. fusca*. Potential mechanisms underlying the associations between *T. fuscum* and *F. fusca*, and how these mechanisms may influence pathogen titer in the insect vector, are discussed.

**Materials and Methods (Pantoea ananatis)**

**Survey of bacteria associated with *F. fusca***: Female *F. fusca* were randomly selected from laboratory colonies (n=64, maintained as described in Chapter 2) and PSREU field populations (n=50, collected as described in Chapter 2). Thrips were rinsed by submerging individuals in successive washes of 0.6% hypochlorite solution, 70% ethanol, and sterile 0.85% saline for 15 s each. After adults were washed, they were dissected in 10 µl of sterile saline to determine parasitism status (as described in Chapter 2). Dissected individual thrips were placed
in a sterile 1.5-ml microcentrifuge tube with 90 µl of saline and sonicated at a setting of 10% for 10 s with a Fisher Sonic Dismembrator microprobe (Model 300, Fisher Scientific, Pittsburgh, PA). An additional 100 µl of saline were added to the tubes, the tubes were vortexed for 10 sec, and 100 µl of the sample were spread onto a tryptic soy broth agar plate (TSBA; 15 g TSB + 7.5 g agar up to 500 ml of water). The remaining 100 µl of sample were stored at 4°C. Plates were incubated at 25°C, and the bacterial phenotypes and number of colonies were recorded after 24 h.

Individual clones were isolated and propagated by transferring single colonies of selected phenotypes to individual plates. Gram staining was performed on the two predominant phenotypes. The isolated bacteria were sent to the University of Florida’s Bacterial Identification and Fatty Acid Analysis Laboratory (Gainesville, FL) for MIDI analysis, a system that uses gas chromatography to identify and compare fatty acid profiles against a bacterial species library.

Statistical analyses were conducted using a logistic regression (Proc GENMOD, SAS 2006) that compared the ratio of thrips harboring bacteria between the different categories of thrips surveyed. Thrips category was defined as the class variable. The genmod procedure allows for unbalanced design and comparison of ratios without data transformation (Neter et al., 1990). Means were compared using the least-square statement of SAS.

Co-infection assay with *P. ananatis* and *T. fuscum*: Adult *F. fusca* were collected from Marion (29°24’ N 82°10’ W) and Alachua (29°38’ N 82°21’ W) County, FL, and maintained in laboratory colonies as previously described (see Chapter 2). The *P. ananatis* stock preparation was obtained by inoculating one single colony of *P. ananatis* to 5 ml of LB broth and vortexing at 150 rpm overnight at 25°C. After 12 hours, the concentration of bacterial solution reached
approximately 5.0 x 10^9 cells/ml. Groundnut leaf discs (1-cm²) were cleaned by successive
washes of 0.6% hypochlorite solution, 70% ethanol, and sterile 0.85% saline for 15 s each.
Discs were dipped into a concentrated \( P.\ ananatis \) stock solution and air dried. A single one-
day-old female was selected randomly from the colony and placed in a 1.5-ml microcentrifuge
tube that contained a leaf disc inoculated with \( P.\ ananatis \). After a 48 h exposure to bacterial-
coated leaf discs, females were transferred either to a 1.5-ml microcentrifuge tube with leaf disc
(controls) or with leaf discs containing two parasitized \( F.\ fusca \) adult females that were excreting
nematodes. Thrips remained in these tubes for an additional 48 h before individuals were
removed, washed as described above, air dried, and individually transferred to a 1.5-ml
microcentrifuge tube with a fresh cleaned leaf disc. The following day, thrips were transferred to
clean tubes. The prior tubes were rinsed with 200 µl of water and the rinsate was examined for
free-living \( T.\ fuscum \) to identify the two parasitized \( F.\ fusca \) that served as an inoculums source
for these assays. Seven and 12 d after the initial exposure to the \( P.\ ananatis\)-treated leaf discs,
individuals were dissected in 25 µl of saline to determine parasitism status, sonicated, and 2 µl of
homogenate spot plated in ten-fold serial dilutions. Resulting colony forming units (CFUs) were
determined after 24 h of incubation at 25°C. Attempts were made to obtain 50 thrips for each
treatment per replicate experiment. The experiment was replicated three times.

For the statistical analyses, colony forming units were long-transformed prior to analysis
and the capability procedure (Proc CAPABILITY; SAS 2006) was used to test for normal
distribution of the transformed data. The frequency procedure (Proc FREQ, SAS 2006) was used
to generate a goodness of fit test and frequency table to determine if the presence of \( P.\ ananatis \)
is associated with parasitism status (Proc FREQ, SAS 2006). Multiple comparison tests among
treatment frequencies were made using Tukey’s style multiple comparison of proportions (Zar,
A mixed model anova (Proc MIXED; SAS 2006) was performed on log-transformed data to test for differences in bacterial CFU counts between parasitism treatments over time. Comparisons between treatment means were made using the LS means option.

**Assays for antimicrobial activity:** Individual *F. fusca* females collected from field populations (see Chapter 2) were dissected in Hepes-buffered saline to determine parasitization status and placed in a 1.5-ml microcentrifuge tube with 50 µl buffer. The two treatments, non-parasitized and parasitized *F. fusca* females, each had two tubes of 50 thrips each. Thrips were homogenized with a plastic pestle on ice, mixed with an additional 50 µl buffer, and centrifuged at 2,000 x g for 3 minutes at 4°C to pellet debris. The supernatant (100 µl) was transferred to a 0.45-µm filtered microcentrifuge tube and centrifuged at 10,000 x g for 5 m. To obtain the acid-derived homogenate, 50 non-parasitized and parasitized *F. fusca* females were dissected and placed in a 2.0-ml conical screw cap microcentrifuge tube (Fisher Scientific, Pittsburgh, PA, USA) with 0.5 ml of 4% acetic acid. Five 2.0-mm Zirconia beads (BioSpec products, Inc., Bartlesville, OK, USA) were added to each of the tubes and samples were homogenized for 20 s using a multi-tube homogenizer (Thermo Savant Fast Prep Homogenizer, Savant, Markham, Ontario). The tubes were individually inspected under a dissecting microscope to ensure thorough homogenization of thrips. Samples were boiled for two minutes to precipitate large molecular weight proteins and centrifuged at 10,000 x g for 10 m to remove heat-denatured proteins. The resulting supernatant was frozen at -70°C overnight and freeze-dried the following day. A tube containing 0.5 ml of 4% acetic acid served as a negative control. After freeze-drying, 40 µl of sterile 0.1M phosphate buffer were added to each tube and sonicated for 10 m to solubilize proteins. The two homogenates were subjected to a series of antimicrobial tests (see below).
The first two antimicrobial tests were conducted to observe if crude or acid-derived homogenates of non-parasitized and parasitized F. fusca females negatively impacted P. ananatis. The antimicrobial tests were conducted by placing 5 µl of the homogenate sample either on a 1-cm² filter disc or in a 2-mm plug on a TSBA plate spread with 200 µl of stock P. ananatis solution. For both antimicrobial tests, streptomycin (2 mg/ml; Sigma, St. Louis, MO) served as a positive control.

The second antimicrobial test was conducted to observe the antibacterial response of F. fusca when parasitized with T. fuscum by determination of lysozyme activity. First, standard chicken egg white lysozyme solution was made in two-fold serial dilution of stock solution (100,000,000 enzyme units (EU)/5g; Cat No. 4403, Calbiochem Merck KGaA, Darmstadt, Germany) to obtain a final protein concentration of 320, 160, 80, 40, 20, 10 and 5 µg/ml or 6400, 3200, 1600, 800, 400, 200 and 100 EU/ml. Two µl of each concentration were placed in individual 2-mm² plugs cut into a Micrococcus lysodeikticus plate (5 mg/ml + 0.1M phosphate buffer + 1.2 g agar; Sigma Chemical, St. Louis, MO). Two µl of the acid derived sample homogenates and 2 µl of a negative control (0.1M phosphate citrate buffer) were also placed in individual 2-mm² plugs and incubated overnight at 37°C. After 24 h, the zone of inhibition for each sample was measured in mm². A standard curve was generated by plotting the zone of inhibition (in mm²) against units of activity. The resulting regression equation was used to determine units of activity per thrips.

The third antimicrobial test was conducted to observe the effect of lysozyme activity on P. ananatis. 200 µl of the bacterial stock solution were spread on a TSBA plate and 2 µl or 15 µl lysozyme standards were placed in plugs or on filter discs on the plate, respectively. Plates were incubated at 37°C and the zone of inhibition recorded after 24 h.
Materials and Methods (*Tomato spotted wilt virus*)

**Viruliferous non-parasitized and parasitized *F. fusca* colonies:** In June 2008, leaves displaying symptoms of TSWV were collected from *A. hypogaea* at the Institute of Food and Agricultural Science Plant and Science Research Education Unit at Citra, FL (29°24’ N 82°10’ W). A 100-mg tissue subsample from each plant was tested for TSWV infection with a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) that uses anti-mouse antibodies to detect viral structural (nucleocapsid) proteins in sample homogenates (SRA 39300, Agdia Inc., Elkhart, IN). Subsamples with an ELISA optical density value greater than the determined threshold value for a plate (mean of control readings + 3[standard deviations]) were considered positive. Plants whose subsample tested positive by ELISA were used to inoculate first instar *F. fusca* as described previously (see Chapter 4). After 72 h, larvae were transferred from TSWV infected plant material to healthy groundnut leaves that were replaced daily until pupation. *Frankliniella fusca* pupae were collected and transferred in groups (n=20) to 1.5-ml microcentrifuge tubes provisioned with a groundnut leaflet. Two *T. fuscum* parasitized females excreting nematodes were added to half of the tubes and two non-parasitized one-day-old females were added to the remaining half of the tubes. Twenty pupae obtained from healthy laboratory colonies were also placed in a 1.5-ml microcentrifuge tube with two non-parasitized adult female *F. fusca* (control). After 24 h, all adult female *F. fusca* were disposed of and individual pupae were placed in tubes with a fresh groundnut disc until adult emergence. The resulting viruliferous treatments were non-parasitized females not exposed to *T. fuscum*, non-parasitized females exposed to *T. fuscum*, and females parasitized by *T. fuscum*. Non-viruliferous, non-parasitized females served as the negative control.

**TSWV titration of *F. fusca* cohorts:** Six-day-old female *F. fusca* were individually dissected in 1µl of Hepes buffered saline and grouped in sterile RNase free 1.5-ml
microcentrifuge tube on ice containing 0.5 ml of TRI Reagent (Sigma-Aldrich Corp., St. Louis, MO, USA) until 20 thrips had been obtained per treatment. The tubes were centrifuged at 5,000 x g for 1 minute 4°C at and stored at -80°C until RNA could be extracted. RNA was extracted from *F. fusca* following the Sigma Technical Bulletin MB-205 for TRI-Reagent (Appendix C1). Reverse transcriptase PCR was conducted on 1 µl RNA from each sample using the Access RT-PCR System (Promega, Madison, WI) following the manufacturer’s Technical Bulletin TB220 protocol. See Appendix C2-C3 for primer information and PCR protocol and cycling profiles for RT-PCR. Five µl of PCR product was run on a 1.5% agarose gel to confirm presence of the TSWV amplicon, and the obtained product was sent to the University of Florida’s DNA Sequencing Core (ICBR) for sequencing to confirm that amplification was of the target gene. RT-PCR was also used to determine suitability of thrips selected internal 28S and COI standards. Reverse transcribed cDNA was synthesized from 50 ng of RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) following the manufacturer’s protocol (Appendix C4). Quantitative real-time PCR (qPCR) was conducted on the reverse-transcribed cDNA using the Quantace iQ SYBR Green Supermix kit (Bioline, Taunton, MA) according to the manufacturer’s protocol (Appendix C5). Following amplification, threshold cycle (Cₜ) values for both the target gene (TSWV) and the internal standard (CO1) were recorded for all samples. Samples whose Cₜ values were above 30 indicated that the target gene was not amplified and were therefore considered negative. The approximate relative quantity of TSWV titer in each sample was calculated by comparative quantification using the equation $2^{-(ΔΔCₜ)}$, where $ΔΔCₜ = (Cₜ_{TSWV} - Cₜ_{CO1})_{calibrator} - (Cₜ_{TSWV} - Cₜ_{CO1})_{unknown}$ (Livak and Schmittgen, 2001).

**TSWV titration of individual *F. fusca*:** The initial assay described above assumed the proportion of thrips in each cohort acquired TSWV equally even though each pool of 20 thrips
may have contained an unknown number of non-viruliferous thrips (i.e., the assay did not provide quantification for which thrips actually harbored virus prior to analysis). Therefore, a second assay was conducted that determined an individual’s vector competence prior to conducting qPCR. To determine the vector competence of individual *F. fusca*, a leaf disc assay similar to that described by Rotenberg et al. (2009) was implemented before thrips were dissected so that the individual’s capacity to transmit TSWV was known. For the leaf disc assay, single one-day-old adult female *F. fusca* were placed individually into 1.5-ml microcentrifuge tubes with a fresh peanut disc (1-cm²). The females were given three consecutive 48 h inoculation access periods (IAP) each with a fresh leaf disc, and the old leaf disc was immediately placed on sterile water in 24-well plates and incubated for 5 d at 25°C to amplify virus titer. Leaf discs were then stored at -80°C for DAS-ELISA. After analysis, thrips from each treatment were categorized into four transmission categories: 0 (0 out of 3 transmissions), 1 (1 out of 3 transmissions), 2 (2 out of 3 transmissions), and 3 (3 out of 3 transmissions). Leaf discs were tested until a minimum of three adults from each treatment filled a transmission category.

After the last IAP, individual females were dissected and their parasitism status was recorded. Females were then transferred to individual tubes with 10 μl of Tri-Reagent™ and stored at -80°C. The Sigma RNA extraction bulletin was optimized for individual thrips and three individuals were randomly selected from each treatment (Microsoft Excel 2007, RAND Function) for analysis by qPCR. An external standard prepared from the pBluescript plasmid containing the TSWV-N gene was used to generate a standard curve for absolute quantification in the qPCR reactions. Serial dilutions ranging from $1 \times 10^6$ to $1 \times 10^1$ plasmid copies per μl were made from the stock plasmid, and $C_T$ values generated from the standard curve were used
to calculate the absolute viral genome copy numbers from individual thrips. Reverse transcribed cDNA from viruliferous thrips tested by qPCR to have a threshold cycle less than 24 served as a positive control. The efficiency of the COI housekeeping gene to serve as a stable internal control was determined by calculating the mean CT values as 2-CT and comparing the fold-change in gene expression between treatments (Schmittgen and Livak, 2008).

**Statistical analyses:** Mean cumulative function plots were generated for viruliferous non-parasitized and viruliferous parasitized female *F. fusca* to observe differences in transmission over the three IAPs (Proc RELIABILITY; SAS, 2006). A generalized linear mixed model (Proc GLIMMIX; SAS, 2006) was used to determine if the overall incidence of transmission was dependent upon parasitism and if parasitism influenced transmission over the three IAPs. Each IAP was treated as a repeated measure, with the measure being a binary response of an infected or non-infected leaf disc. Pairwise comparisons among the treatment groups were made using the LS means option. A general linear anova model (Proc GLM, SAS 2006) was performed on log transformed copy numbers to determine if viral copy number is dependent upon *F. fusca* parasitism status and/or the number of transmission events by viruliferous thrips. The model was extended for an ordinal logistic regression (Proc LOGISTIC, SAS 2006) to determine whether the probability of transmission was related to copy number or parasitism status. A scatter plot and line of best fit was generated to determine if a linear correlation existed between ELISA optical density values and TSWV copy numbers.

**Results (Pantoea ananatis)**

Survey of bacteria associated with *F. fusca*: Both *F. fusca* laboratory and field individuals contained bacteria cultivable on TSBA (Table 5-1). Transmission electron micrographs revealed the bacteria are located within the midgut and hindgut of individual thrips, and in close
apposition to the midgut microvilli and the hindgut intimal lining (Figure 5-1). The bacteria are fairly uniform in shape (1 µm in diameter), with both short and long rods present.

Two bacterial phenotypes were frequently found on the plated individuals at 24 h post-inoculation (Figure 5-2A). Phenotype A was identified on TSBA as smooth, yellowish, dry, and circular umbonate colonies 1.5-mm² in diameter with compact, raised centers and flattened smooth edges (Figure 5-2B). Phenotype B was identified on TSBA by its shiny, wet appearance and milky white texture (Figure 5-2C). The proportion/percentage of *F. fusca* harboring cultivable bacteria was higher for non-parasitized (x=78%) than for parasitized (x=61%) females collected from either the field or laboratory populations (Table 5-1). Statistically, non-parasitized *F. fusca* collected from laboratory populations had a significantly higher frequency of individuals with cultivable bacteria than any other category ($\chi^2=14.75$, d.f. =1, P<.0001). Over 90% of individuals that harbored bacteria contained phenotype A, and the frequency of individuals with this phenotype was statistically higher for laboratory colonies of non-parasitized *F. fusca* ($\chi^2=11.65$, d.f.=1, P=.0006) than for the other populations ($\chi^2=704.07$, d.f.=1, P<.0001) (Table 5-1). Only 15% of the *F. fusca* tested contained phenotype B, and over half these individuals were collected from non-parasitized field populations (Table 5-1). Non-parasitized and parasitized *F. fusca* were rarely infected with any other bacterial phenotype (Table 5-1). Co-infection of two or more phenotypes was not common (Table 5-1).

Both the A and B bacterial phenotypes were identified as Gram-negative. According to MIDI analysis, the fatty acid profile of phenotype A had a high association to *P. ananatis/Erwinia uredovora* (Sim Index = 0.905). However, the less frequent phenotype B was not identified with MIDI analysis; the GC profile had a low association to both *Ewingella americana* (Sim Index = 0.224) and to *Pantoea agglomerans* (Sim Index = 0.150; see Appendix
C6 for the full MIDI report). Phenotype A (*P. ananatis*) was selected for the co-infection assays with *T. fuscum* due to the high frequency of individuals associated with this phenotype.

Co-infection assay: In an attempt to increase the number of *F. fusca* females harboring *P. ananatis*, adults were exposed for 48 h to bacterial-coated leaf discs. This exposure period increased the number of thrips with bacteria from 66% to 75% (Table 5-1, 5-2). Exposure to *T. fuscum* did not affect the number of non-parasitized *F. fusca* harboring *P. ananatis* (Table 5-2). However, there was a statistically significant reduction in the number of parasitized *F. fusca* acquiring *P. ananatis* when compared to the non-parasitized conspecifics ($\chi^2=39.73$, d.f. =2, $P<.0001$). There was a 40% reduction in the number of parasitized *F. fusca* females harboring *P. ananatis* when compared to the non-parasitized treatments (Table 5-2).

The number of bacterial CFUs recorded for non-parasitized *F. fusca* ranged from 0 – 1.7 x $10^8$. The number of bacterial CFUs recorded for parasitized *F. fusca* ranged from 0 – 7.0 x $10^6$. The CFUs were log-transformed prior to analysis to achieve a more normal distribution of the data. Statistically, there was a significant difference in the CFU counts among all thrips ($F_{2,296}=20.97$, $P<0.0001$), with the parasitized *F. fusca* having a lower number of CFUs than either the non-parasitized ($t=5.41$, d.f. = 296, $P<.0001$) or the exposed, non-parasitized ($t=-5.84$, d.f. =296, $P<.0001$) (Table 5-2). There was not a statistically significant difference in CFU counts between the control and exposed, non-parasitized *F. fusca* females ($t=-0.34$, d.f. =296, $P=0.73$) (Table 5-2). The length of time *F. fusca* incubated *P. ananatis* did not significantly influence the number of CFUs for all three treatments ($F_{1,296}=2.08$, $P=0.15$) (Table 5-2). Although not statistically significant, the non-parasitized *F. fusca* exposed to *T. fuscum* had nearly twice as many CFUs when they harbored *P. ananatis* for five additional days ($t=-2.21$, d.f.=296, $P=0.03$).
Antimicrobial assays: Crude and acid-derived homogenates of non-parasitized and parasitized *F. fusca* did not exhibit antimicrobial activity against *P. ananatis* (Fig 5-3a, b). Lysozyme activity was not detected in non-parasitized female *F. fusca*, but parasitized *F. fusca* females exhibited lysozyme activity against *M. lysodeikticus* after 24 hours that resulted in a zone of clearance equaling 28.3 mm². From the standard curve, the lysozyme activity of this sample was calculated to be equal to 2.88 EU per single thrips (Figure 5-3c, Figure 5-4, Table 5-3). The lysozyme standards did not show activity against *P. ananatis* (Fig 5-3d).

Results (TSWV)

TSWV titration of *F. fusca* cohorts: Reverse transcriptase PCR validated the presence of all targeted products (Figure 5-5). The COI primers amplified a fragment of the expected size (143 bp) for all samples and the TSWV primers amplified a fragment of the expected size (123 bp) for all viruliferous samples. TSWV product was not detected in the non-viruliferous cohorts. The 28S primers amplified negative controls and were therefore not used for further PCR (Figure 5-6). Sequencing results of the targeted gene confirmed amplification was of the TSWV N-protein (Appendix 5-7).

Quantitative PCR of cDNA synthesized from total extracts of viruliferous female *F. fusca* detected COI and TSWV – N protein transcripts in the appropriate samples (Figure 5-7A,B). Amplification of the TSWV-N protein was not observed in non-viruliferous samples within 30 cycles (Figure 5-7C). The average $C_T$ values for the three treatments are located in Table 5-4. The average $C_T$ values for the COI transcripts (internal control) varied by only one threshold cycle between all four treatments, indicating extraction efficiencies were similar between all sample cohorts. There was variation in viral titers both between and within all the viruliferous treatments, as indicated by the high standard deviations associated with the average TSWV $C_T$ values. The largest variation in viral titer was observed with the viruliferous parasitized *F. fusca*.
cohorts, where average C_T values ranged from 36.48 ± 1.31 to 22.25 ± 0.17 (data not shown). Overall, there was a 33-fold and 124-fold reduction in the TSWV titers of parasitized _F. fusca_ females when compared to the non-parasitized and _T. fuscum_ exposed (non-parasitized) cohorts, respectively. There was a 3-fold increase in virus titer for _F. fusca_ exposed to _T. fuscum_ (non-parasitized) compared to those not exposed.

**TSWV titration of individual _F. fusca_:** Of the 980 _F. fusca_ assayed to estimate TSWV titer, 795 and 185 of the thrips eclosed as females and males, respectively (data not shown). Nearly one third of the adults (n=285) died before the end of the assay, the majority of which (75%) succumbed to infection by _Entomophthorales_ sp. during the last IAP. A total of 676 thrips survived throughout all three IAPs, and dissections of these individuals revealed there were nearly twice as many non-parasitized than parasitized _F. fusca_. Only 31 of thrips surviving through the last IAP were infected with _Entomophthorales_ sp.. Overall, 1,218 leaf discs were analyzed by DAS-ELISA from the non-parasitized (N=262) and parasitized (N=144) _F. fusca_ females.

The DAS-ELISA absorbance values for each leaf disc were used to categorize non-parasitized and parasitized _F. fusca_ according to their vectoring capabilities. Parasitism by _T. fuscum_ reduced the proportion of transmitting individuals by 20% (Table 5-5). On average, 74 and 54% of non-parasitized and parasitized female _F. fusca_, respectively, transmitted TSWV at least one time during the three consecutive IAPs (Table 5-5). Of the 406 _F. fusca_ individuals tested by leaf disc assay, only 47 transmitted TSWV during all three IAPs (Table 5-5). The mean cumulative frequency of TSWV transmission was significantly higher over all three IAPs for non-parasitized thrips (F_1,465=19.80, P<.0001) (Figure 5-8, Table 5-5). The rate of transmissions varied over the three IAPs (F_2,786=23.77, P<.0001), with transmission being
significantly lower in the first IAP than in the second (t=-6.14, d.f.=755, P<.0001) and third (t=-6.27, d.f.=1195, P<.0001) IAPs for both non-parasitized and parasitized *F. fusca* (Table 5-6). Non-parasitized individuals transmitted at a significantly higher frequency than parasitized *F. fusca* over the second (F1,392=9.24, P=.0025) and third (F1,388=9.82, P=.0019) IAP (Table 5-6). Statistically, there was no significant difference in IAP of parasitized *F. fusca* transmitting TSWV (F2,786=2.79, P = 0.062) (Table 5-6). However, the efficiency of both parasitized and non-parasitized thrips transmitting TSWV to leaf discs was higher during the third IAP than any other period (Table 5-6).

Optimization of methods used for RNA extraction from viruliferous thrips and the generation of the standard curve for qPCR is shown elsewhere (see Appendix C8-C9). The Cₜ values of non-parasitized and parasitized thrips for the COI housekeeping gene did not vary significantly between samples under conditions of the qPCR signifying its suitability as a stable internal control (Appendix 5-6). Despite the optimization methodologies used for extracting RNA from individual *F. fusca*, TSWV and COI copy numbers were extremely low and variable across all treatments (Table 5-7). The COI and TSWV Cₜ values across treatments ranged from 12.41 to 23.58 and 21.09 to 37.39, respectively. Some comparisons in absolute quantification were able to be made between the different treatments of *F. fusca* despite the variable extraction efficiencies. Non-transmitting *F. fusca* had significantly fewer genome copy numbers of TSWV than those that transmitted TSWV regardless of parasitism status (Figure 5-9). The TSWV copy number increased with the number of transmissions for both non-parasitized and parasitized *F. fusca* (F3,79=2.78, P=.0465). Non-parasitized *F. fusca* had higher TSWV titers than parasitized thrips, but this was not found to be significant (F1,76=3.75, P=0.0567) (Figure 5-10). The logistic regression, which evaluated the number of transmissions as a function of parasitism status and
copy number, found copy number to be positively correlated with the number of transmissions ($\chi^2=6.25$, d.f.=1, $P=.01$) and parasitism status to not affect the number of transmissions to leaf discs ($\chi^2=0.28$, d.f.=1, $P=.60$). There was no statistically significant correlation between ELISA optical density values and TSWV viral copy numbers ($R^2=0.0138$) (Figure 5-11).

**Discussion**

In this study, extracellular bacteria observed in the mid- and hindgut of individual *F. fusca* were similar to thrips gut bacteria reported by others (McKenzie et al., 1993; Wells et al., 2002, Gitaitis et al., 2003; Ullman et al., 1989; de Vries et al., 1995, 2001, 2004, 2008; Chanbusarakum and Ullman, 2008, 2009). *Pantoea ananatis* was the dominant bacterium isolated from over 90% of individual *F. fusca* collected from laboratory and field populations. The high association of *P. ananatis* with individual *F. fusca* collected over space and time suggests a symbiotic relationship between the bacteria and host. Similarly, Chanbusarakum and Ullman (2008, 2009) found related strains of *Enterobacteriaceae* in *F. occidentalis* collected from geographically isolated areas and suggested the bacteria and hosts share a facultative symbiotic association. In an attempt to clarify the relationship of gut bacteria on thrips host, de Vries et al. (2004) examined fitness effects of *F. occidentalis* with and without bacteria. By feeding different diets to the thrips, they discovered thrips with gut bacteria have reduced fitness effects with a nutrient-rich diet and increased fitness effects with a nutrient-depleted diet. These results suggest the gut bacteria are both parasitic and mutualistic by either competing with the host under favorable environments or benefiting their hosts in poor environments.

Environmental conditions can also influence the phenotypic characteristics of bacteria, including behavior, growth, and biofilm formation (Brown and Barker, 1999). These characteristics can impact the prevalence of bacteria growth on a host. *Frankliniella fusca* collected from laboratory colonies had the highest frequency of individuals infected with *P.*
ananatis and highest CFU counts (data not shown). Transmission of P. ananatis occurs horizontally on the food source from feces or saliva or vertically through contamination of the egg shell (de Varies et al., 2001b; Wells et al., 2002). The environmental conditions for rearing F. fusca in laboratory colonies are more suitable for P. ananatis growth and transmission than are field conditions (see Chapter 2). Laboratory conditions include a high humidity and temperature, a shared food substrate, and a high density of thrips maintained over subsequent generations. Individual F. fusca collected from field populations are restricted to contacting relatively few isolated thrips populations located in groundnut flowers or terminal buds.

Parasitism by T. fuscum also influenced the number of individuals infected with P. ananatis and reduces the quantity of bacteria in the host insect as demonstrated by the co-inoculation assays. There was not a statistically significant difference in the quantity of bacteria in parasitized F. fusca individuals over time, suggesting the parasitism event by the motherworm is responsible for reducing P. ananatis in the thrips host. It is unlikely the motherworm residing in the hemocoel exerts a direct effect on bacteria in the alimentary tract. Infection by certain microbes may indirectly induce a host immune response that acts against pathogens in the insect vector. Acid-derived homogenates of parasitized F. fusca exhibited lysozyme activity against M. luteus but activity by the lysozyme standard did not inhibit P. ananatis. The high concentration of P. ananatis spread onto the TSBA plates likely concealed any detectable activity by the lysozyme standard (i.e., the concentration of the bacteria on the plate was much higher than would naturally occur in the thrips). Lysozyme is commonly known as an antibacterial enzyme of the insect immune system synthesized by insect fat body and hemocytes upon infection with bacteria, fungi, viruses, nematodes, etc. (Bulet et al., 1999). The detection of lysozyme in the present study may suggest immune up-regulation, but parasitism by T. fuscum does not decrease
the lifespan (an indicator of upregulation) of *F. fusca* (Sims et al., 2005). Lysozyme also functions as a digestive enzyme in the midgut and salivary glands of some insects by lysing gut bacteria and providing insects with additional nutrients (Callewaert and Michiels, 2010). The increased lysozyme concentrations observed in parasitized *F. fusca* may be a host-induced response to digest and sequester nutrients from *P. ananatis* since *T. fuscum* reduces feeding of infected individuals (see Chapter 4).

Transmission assays in this study validated previous results that *T. fuscum* reduces the capacity of *F. fusca* to vector TSWV (see Chapter 4). Differences in transmission rates between non-parasitized and parasitized *F. fusca* in the two experiments are likely due to the different IAPs utilized (48 vs. 24 h). Nonetheless, parasitism by *T. fuscum* reduced transmission rates of *F. fusca* even with a longer IAP. Previous results also suggested that *T. fuscum* may induce a direct effect on TSWV replication (see Chapter 4), and assays conducted to quantify viral genome titers found significant variation between all viruliferous *F. fusca* samples in both the cohort and individual qPCR assays. Viral titer in insect vectors is known to vary and is thought to reflect different physiological potentials between individuals to amplify virus in the salivary glands (Ullman et al., 1993; Wijkamp et al., 1995; Nagata et al., 1999). However, transcription of the COI housekeeping gene should not have varied between replicate samples in the individual qPCR assays. This variation, in combination with low TSWV yield, suggests possible degradation of thrips samples stored long-term at -80°C in TRI-Reagent™. Bravo et al. (2007) found tissue preserved in RNA Later™ at -80°C, followed by RNA extraction and storage at -80°C, to be unstable for small RNAs (20 nt) when using Trizol™ reagent as an isolation method. In contrast, Mraz et al. (2009) reported that TRI-Reagent™ based isolation produced stable RNA suitable for long-term storage at -80°C. In this experiment, an alternative RNA
extraction method may have yielded higher extraction efficiency. Boonham et al. (2002) reported detection of TSWV in viruliferous *F. occidentalis* to be more efficient when using a chelating resin rather than alcohol-precipitation for RNA extraction. Using this method, Rotenberg et al. (2009) were able to obtain $2 \times 10^5$ - $2 \times 10^7$ copies of N transcript per female *F. occidentalis*.

Regardless of the low extraction efficiency, dose-dependency was found between viral copies and numbers of transmissions. These findings corroborate other reportings that TSWV present in viruliferous thrips is a quantitative factor affecting vector competence (Nagata, 2002, Wijkamp et al., 1995; van de Wetering et al., 1996; Rotenberg et al., 2009). Wijkamp et al. (1995) and Nagata et al. (1999) found that small amounts of virus were detected in the salivary glands of non-transmitting thrips, which suggests there is a threshold titer of TSWV required for thrips to transmit successfully. In this experiment, low viral titers were also detected in non-transmitting *F. fusca* and higher titers tended to be associated with transmission.

Results from this study demonstrate that parasitism by *T. fuscum* is a qualitative factor affecting vector competence of host thrips. While in the cohort assay parasitized *F. fusca* females had a significantly lower titer of TSWV than non-parasitized females, parasitism was found to be a non-significant factor impacting viral titer for the individual assay. It should be noted that the pooled thrips in the cohort samples were completely randomized and the chance of selecting a combined sample of 20 non-viruliferous from virus-exposed thrips in the experimental pools was very unlikely. Individual female *F. fusca* exposed to TSWV and parasitized by *T. fuscum* were more likely to not transmit TSWV when compared to their non-parasitized conspecifics (Table 5-5). It is more probable that parasitized individuals pooled in
the cohort assay had a reduced TSWV titer because they had a higher percentage of non-viruliferous thrips.

The response of the *F. fusca* vector to the plant pathogens *P. ananatis* and TSWV is modulated by its interaction with *T. fuscum*. To my knowledge, this is the first report of infection by an obligate parasite antagonistically affecting a bacterial and viral pathogen in an insect vector. It is unknown whether the reduction in plant pathogens is induced by the *F. fusca* host, the parasitic *T. fuscum*, or is simply a ‘by-product’ of infection (Poulin, 1995; Adamo, 2002; Thomas et al., 2005). In both the *P. ananatis* and TSWV coinfection assays, *F. fusca* acquired the plant pathogen before acquiring the *T. fuscum* parasite. Possibly, primary infection by the pathogen activated the host immune system (as shown by Medeiros et al., 2004) and the enhanced immune response under dual infection with the parasitic *T. fuscum* reduced and/or eliminated the plant pathogen but not the parasite. Data from Chapter 3 suggests *T. fuscum* evades host immunity as demonstrated by the lack of cellular and humoral immune factors at the host-parasite interface. Alternatively, the parasitic *T. fuscum* may outcompete plant pathogens in the insect vector by (1) releasing antimicrobial metabolites that are active against TSWV and *P. ananatis*; (2) sequestering available host nutrients; (3) inhibiting normal host alimentary and salivary gland function; or (5) interfering with other host endosymbionts that regulate host vector capacity.

As reviewed in the introduction, most of the research conducted on insect vector-pathogen-microbe systems suggests immune activation is an important factor in regulating infection. It is most likely that the parasitism event by the *T. fuscum* motherworm activates the transcriptional upregulation of the thrips immune system, which in turn, triggers a cascade of antibacterial and antiviral peptides that act against both pathogens. Most work on insect immunity comes from
molecular studies on *Drosophila melanogaster* where researchers have discovered the induction of innate antibacterial (pattern recognition receptor proteins) and antiviral (RNA interference and silencing) mechanisms upon parasite infection (Schmid-Hempel, 2005; Kemp and Imler, 2009; Sabin et al., 2010). Further studies like those conducted by Medeiros *et al.* (2004), who used subtractive cDNA libraries to examine the upregulation of *F. occidentalis* gene activity upon TSWV infection, will be needed for validation of this hypothetical mechanism (see Chapter 6). Elucidating the mechanisms behind how *T. fuscum* disrupts the plant pathogens *P. ananatis* and TSWV within the *F. fusca* vector may offer novel biological-based management strategies for controlling plant disease spread by Thysanoptera (see Chapter 6).
Table 5-1. The percentage of individual females containing bacteria cultivable on tryptic soy broth agar plates from non-*T. fuscum* parasitized and *T. fuscum* parasitized *Frankliniella fusca* laboratory and field populations.

<table>
<thead>
<tr>
<th>Location</th>
<th><em>F. fusca</em> with bacteria</th>
<th><em>F. fusca</em> with bacteria harboring phenotype A</th>
<th><em>F. fusca</em> with bacteria harboring phenotype B</th>
<th><em>F. fusca</em> with bacteria harboring other phenotypes</th>
<th><em>F. fusca</em> with bacteria infected with &gt;1 phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-parasitized <em>F. fusca</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory (n=39)</td>
<td>85% (33)</td>
<td>97% (32)</td>
<td>6% (2)</td>
<td>0</td>
<td>3% (1)</td>
</tr>
<tr>
<td>Field (n=34)</td>
<td>71% (24)</td>
<td>79% (19)</td>
<td>33% (8)</td>
<td>4% (1)</td>
<td>13% (3)</td>
</tr>
<tr>
<td>Parasitized <em>F. fusca</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laboratory (n=25)</td>
<td>56% (14)</td>
<td>100% (14)</td>
<td>7% (1)</td>
<td>0</td>
<td>7% (1)</td>
</tr>
<tr>
<td>Field (n=16)</td>
<td>69% (11)</td>
<td>91% (10)</td>
<td>9% (1)</td>
<td>9% (1)</td>
<td>9% (1)</td>
</tr>
</tbody>
</table>

The number in parentheses represents the number of *F. fusca* within each category.

Table 5-2. The mean number of *Pantoea ananatis* colony forming unit counts ± standard deviation for early and late stage *Frankliniella fusca* plated individuals, based on *Thripinema fuscum* parasitism status.

<table>
<thead>
<tr>
<th>Treatment (314)</th>
<th>7 days after exposure to <em>P. ananatis</em></th>
<th>12 days after exposure to <em>P. ananatis</em></th>
<th>% of <em>F. fusca</em> with <em>P. ananatis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-parasitized <em>F. fusca</em> (102)</td>
<td>1.8 x 10^5 ± 1.06 x 10^4 a</td>
<td>1.0 x 10^6 ± 1.76 x 10^6 a</td>
<td>85% (87)</td>
</tr>
<tr>
<td>Parasitized <em>F. fusca</em> (99)</td>
<td>2.5 x 10^5 ± 1.05 x 10^6 b</td>
<td>1.5 x 10^5 ± 2.81 x 10^5 b</td>
<td>52% (51)</td>
</tr>
<tr>
<td>Exposed, non-parasitized <em>F. fusca</em> (97)</td>
<td>1.9 x 10^6 ± 7.5 x 10^6 a</td>
<td>4.5 x 10^6 ± 2.39 x 10^7 a</td>
<td>86% (113)</td>
</tr>
</tbody>
</table>

Numbers within the column followed by the same letter are not significantly different according to least squares mean (p≥0.05). Numbers in parentheses corresponds to the number of individuals within each category.
Table 5-3. Measurements of the zone of inhibition (in mm) for lysozyme standards (µg/ml) used for determining the units of activity (EU/ml) in acid-derived *T. fuscum* parasitized *Frankliniella fusca* homogenates.

<table>
<thead>
<tr>
<th>stock (µg/ml)</th>
<th>Zone of inhibition (mm)</th>
<th>Units of activity (EU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td>9</td>
<td>6400</td>
</tr>
<tr>
<td>160</td>
<td>8</td>
<td>3200</td>
</tr>
<tr>
<td>80</td>
<td>7</td>
<td>1600</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>800</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>400</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>200</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 5-4. The average threshold cycle (C<sub>T</sub>) values ± standard deviation for TSWV and COI transcripts and relative quantity of TSWV detected by quantitative PCR for viruliferous *Frankliniella fusca* cohorts.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average C&lt;sub&gt;COI&lt;/sub&gt;</th>
<th>Average C&lt;sub&gt;TSWV&lt;/sub&gt;</th>
<th>ΔC&lt;sub&gt;T&lt;/sub&gt;</th>
<th>ΔΔC&lt;sub&gt;T&lt;/sub&gt;</th>
<th>Relative increase*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-viruliferous non-parasitized <em>F. fusca</em></td>
<td>12.75 ± 0.58</td>
<td>35.9 ± 1.6</td>
<td>23.15</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>Viruliferous non-parasitized <em>F. fusca</em></td>
<td>13.11 ± 0.53</td>
<td>22.06 ± 3.53</td>
<td>8.95</td>
<td>-14.20</td>
<td>18820</td>
</tr>
<tr>
<td>Viruliferous <em>F. fusca</em> exposed to <em>T. fuscum</em></td>
<td>13.08 ± 0.23</td>
<td>20.11 ± 1.87</td>
<td>7.03</td>
<td>-16.12</td>
<td>71275</td>
</tr>
<tr>
<td>Viruliferous parasitized <em>F. fusca</em></td>
<td>13.77 ± 0.63</td>
<td>27.76 ± 7.64</td>
<td>13.99</td>
<td>-9.16</td>
<td>573</td>
</tr>
</tbody>
</table>

*Expressed as a number-fold difference to the control.
Table 5-5. Efficiency of *Tomato spotted wilt virus* transmission by non-*T. fuscum* parasitized and *T. fuscum* parasitized *Frankliniella fusca* individuals.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of transmissions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Non-parasitized females (n=262)</td>
<td>25.6% (67)</td>
</tr>
<tr>
<td>Parasitized females (n=144)</td>
<td>46.53% (67)</td>
</tr>
</tbody>
</table>

Numbers in parentheses corresponds to the number of individuals within each category.

Table 5-6. The percentage ± standard error of viruliferous *Frankliniella fusca* cohorts transmitting *Tomato spotted wilt virus* during each inoculation period.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Inoculation Access Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 (0-48 hr)</td>
</tr>
<tr>
<td>Non-parasitized females (n=263)</td>
<td>25.8 ± 2.7a</td>
</tr>
<tr>
<td>Parasitized females (n=145)</td>
<td>21.1 ± 3.4ad</td>
</tr>
</tbody>
</table>

Percentages within the columns followed by the same letter are not significantly different according to differences of LS means (P<0.05).
Table 5-7. The average threshold cycle (Ct) values for TSWV and COI transcripts detected by quantitative PCR for individual viruliferous *Frankliniella fusca*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average CT&lt;sub&gt;COI&lt;/sub&gt;</th>
<th>Average CT&lt;sub&gt;TSWV&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-parasitized <em>F. fusca</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (12)</td>
<td>16.72 ± 1.89</td>
<td>34.27 ± 3.36</td>
</tr>
<tr>
<td>1 (15)</td>
<td>17.36 ± 2.25</td>
<td>30.3 ± 3.73</td>
</tr>
<tr>
<td>2 (19)</td>
<td>16.35 ± 1.66</td>
<td>28.91 ± 2.95</td>
</tr>
<tr>
<td>3 (6)</td>
<td>16.73 ± 3.40</td>
<td>28.84 ± 4.39</td>
</tr>
<tr>
<td>Parasitized <em>F. fusca</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 (11)</td>
<td>17.58 ± 2.20</td>
<td>32.87 ± 3.88</td>
</tr>
<tr>
<td>1 (13)</td>
<td>17.14 ± 1.63</td>
<td>30.92 ± 3.79</td>
</tr>
<tr>
<td>2 (12)</td>
<td>17.42 ± 2.05</td>
<td>30.81 ± 2.53</td>
</tr>
<tr>
<td>3 (6)</td>
<td>17.82 ± 3.40</td>
<td>30.07 ± 5.63</td>
</tr>
</tbody>
</table>

The number in parentheses corresponds to the number of individuals within each category.
Figure 5-1. Transmission electron micrographs of the alimentary tract of a non-parasitized and *Thripinema fuscum* parasitized *Frankliniella fusca* female. (A) The midgut and (B) hindgut lumen of a non-*Thripinema fuscum* parasitized female; (C) The midgut and (D) hindgut of a *T. fuscum* parasitized female. Note the direct association of bacteria to the microvilli of midgut epithelial cells.
Figure 5-2. Bacteria isolated from *Frankliniella fusca* individuals. (A) Bacterial colonies of various bacteria isolated from *F. fusca* individuals growing on a Tryptic soy broth agar plate; (B) Phenotype A at 1.6X magnification; (C) Phenotype B at 1.6X magnification. Scale bar = 10 mm.
Figure 5-3. A series of assays testing for antimicrobial activity from non-\textit{Thripinema fuscum} parasitized and \textit{T. fuscum} parasitized \textit{Frankliniella fusca} individuals against \textit{Pantoea ananatis}. (A) A crude and (B) acid-derived homogenates tested against \textit{P. ananatis}; (C) Acid derived homogenates tested against \textit{Micrococcus lysodeikticus}; (D) lysozyme standards tested against \textit{P. ananatis}. s=streptomycin; c=buffer control; np=non-parasitized \textit{F. fusca}; p=parasitized \textit{F. fusca}. 
Figure 5-4. The standard curve used to generate units of activity for acid-derived *Thripinema fuscum* parasitized *Frankliniella fusca* homogenates.
Figure 5-5. Reverse transcriptase PCR product of *Frankliniella fusca* cohort extractions using TSWV and COI primers. Lane 1=Marker Hyperladder II (5 µl), lanes 2,6 = non-viruliferous non-*Thripinema fuscum* parasitized *F. fusca*, lanes 3,7 = viruliferous non-*T. fuscum* parasitized *F. fusca*, lane 4,8 = viruliferous non-*T. fuscum* parasitized *F. fusca* exposed to *T. fuscum*, lane 5,9 = viruliferous *T. fuscum* parasitized *F. fusca*, lane 10 = TSWV negative control, lane 11 = COI negative control.

Figure 5-6. PCR product testing the suitability of using 28S as an internal control for *Frankliniella fusca* RNA extractions. Lane 1=Marker Hyperladder II (5 µl), lane 2=viruliferous non-*Thripinema fuscum* parasitized *F. fusca*, lane 3=viruliferous parasitized *F. fusca*, lane 4=healthy *A. hypogaea* plant tissue, lane 5=TSWV+ *A. hypogaea* plant tissue, lane 6=negative control (TSWV primer), lane 7=negative control (28S primer). Loading volume was 5 µl of PCR product.
Figure 5-7. An amplification plot from a cohort qPCR reaction. The graph shows the detection of (A) COI and the TSWV N-protein for (B) viruliferous and (C) non-viruliferous *Frankliniella fusca*.

Figure 5-8. The mean cumulative frequency (MCF) of *Tomato spotted wilt virus* (TSWV) transmissions for non-*Thripinema fuscum* parasitized and *T. fuscum* parasitized *Frankliniella fusca* females for each inoculation access period (IAP).
Figure 5-9. A box plot of the number of *Tomato spotted wilt virus* (TSWV) copy numbers (data log-transformed) according to the *Thripinema fuscum* parasitism status of viruliferous *Frankliniella fusca* females.

Figure 5-10. A scatter plot of the correlation between *Tomato spotted wilt virus* (TSWV) copy number and ELISA optical density (OD$_{405}$) values for *Frankliniella fusca* females.
The F. fusca/T. fuscum system is an ideal model for examining the interface between a host insect and its obligate parasite. Thripinema has a direct life cycle that does not require an intermediate host; therefore, all physiological development occurs inside the thrips. Unlike vertebrate-parasitizing nematodes, which develop within host tissues, all Thripinema life stages exist in the insect hemocoel and can be readily collected. Thripinema, unlike other entomopathogenic nematodes, depends upon its host for survival and transmission and has negligible effects on thrips longevity or mortality. It is important to emphasize that all species of thrips known to be parasitized by Thripinema are considered pests, and all Thripinema spp. appear to induce similar impacts on their host thrips; thus, the results of this project can be extended to these systems.

In Chapter 2, I used Wolbachia specific 16S rRNA primers to identify whether Wolbachia strain(s) are associated with non-parasitized female and male F. fusca, T. fuscum parasitized F. fusca females, and free-living T. fuscum nematodes collected over time and space. I proceeded with a multigene approach (wsp, gatB, coxA, hcpA, ftsZ, fbpA) to strain type the Wolbachia(s) associated with six select population cohorts of non-parasitized F. fusca females. All populations tested were associated with one or more Wolbachia strains. I additionally determined Wolbachia impacted the arrhenotokous F. fusca population biology through a series of developmental and reproductive bioassays involving Wolbachia-infected and Wolbachia-cured individuals. Transmission electron micrographs documented Wolbachia infection in the reproductive tissues of non-parasitized and T. fuscum parasitized F. fusca females. However, the discovery of Wolbachia in this system leaves many unanswered questions regarding the tri-trophic interaction between the endosymbiont, thrips, and nematode parasite. For example, what
are the effects of Wolbachia on the fitness parameters of F. fusca and T. fuscum? Results from the mating bioassays suggest Wolbachia alters host reproduction. Further studies involving mating experiments between tetracycline-treated females and males (e.g., Wolbachia-cured females mated with infected and non-infected males and vice versa) are warranted to elucidate the full impact of Wolbachia on host reproduction. Studies evaluating the presence of Wolbachia on host vector competence are also needed. In order to determine the effects of Wolbachia on T. fuscum, the parasite must survive in the absence of Wolbachia; however Wolbachia is an obligate symbiont of filarial nematodes and antibiotic treatment results in mortality of the filarial parasite. It would be interesting to observe whether antibiotically-treated parasitized F. fusca induced host mortality or cleared the host of the T. fuscum parasites and/or restored their reproductive potential. One potential pitfall for these experiments is the need to develop genetically-identical colonies of F. fusca, Wolbachia, and T. fuscum so the researcher knows the exact Wolbachia strain(s) utilized in the laboratory (vs. genetically diverse field collections). Regardless, manipulating Wolbachia in F. fusca may affect vector competence, and as a result, offers potential as a biological control agent (e.g., removing Wolbachia in F. fusca females results in the production of all male progeny, Wolbachia may upregulate the immune system in infected thrips thus reducing in vivo plant pathogens, etc.).

In Chapter 3, I conducted a thorough histological examination of non-parasitized female F. fusca, T. fuscum parasitized female F. fusca, and T. fuscum nematodes. Light and electron microscopy revealed significant internal damage to the fat body and reproductive structures of parasitized F. fusca. Based on observations of the host systems and tissues, I hypothesize that the invasive motherworm releases effector molecules that block egg formation in the thrips panoistic ovary, down-regulates vitellogenesis in the fat body, destructs the follicular epithelium
(apoptosis), and/or or induces hormonal dysfunction. Results from data in Chapter 3 also suggest *T. fuscum* either evades or suppresses the host thrips immune system.

In Chapters 4 and 5, I report that *T. fuscum* reduces host feeding and suppresses the vector competency their thrips host by altering host behaviors and by reducing their ability to maintain plant pathogens *in vivo*. The major impacts of parasitism affecting host function (*i.e.*, reductions in feeding and vector competency) are induced by the single invasive female prior to production of first-generation progeny nematodes. Therefore, I hypothesize that the parasitic motherworm interferes with host physiology by producing effector molecules, or “metabolites” that disrupt host metabolism, are detrimental to viruses and bacteria (*e.g.*, antimicrobials), disturb pathways involved in host behaviors, and/or signal the up-regulation of the thrips innate immune system. Again, data from these two chapters support the hypothesis that an upregulation of the host immune system upon dual infection is active against plant pathogens but not the *T. fuscum* parasite.

Data from this project suggest the upregulation of the thrips immune system is a key factor in regulating entomoparasites in the *F. fusca* vector when parasitized by *T. fuscum*. New technologies involving high-throughput sequencing technology, in combination with microarray analysis, would elucidate the interplay between the thrips and its obligate parasite *T. fuscum* and identify key gene and pathways that are specifically up- and down-regulated by *T. fuscum*. The genes and/or pathways associated with host tissues altered by parasitism may provide additional targets for designing experiments that address TSWV acquisition and transmission.

Presently, it is unknown the mechanisms *Thripinema* utilizes to affect the behavior of *F. fusca*. Lack of such knowledge precludes understanding as to how this host-parasite system interfaces with TSWV disease epidemiology and hinders successful implementation of this
important biological control agent. I hope the research I have conducted during my time at the University of Florida opens the door to a new, exploratory avenue for controlling Thysanoptera and the pathogens they spread.
APPENDIX A
WOLBACHIA 16S RNA, COI, AND MLST SEQUENCES

A1. Wolbachia specific 16S rRNA nucleotide sequences for 28 non-parasitized Frankliniella fusca, parasitized F. fusca, and Thripinema fuscum cohort populations.

>HF1_UF_09
GTGTTGCATGGCTGTCGTCAGCTCGTGCAGTCTAGTTGGTGGTTAAGTCCCGCAACG
AGCGCAACCTCATCCTTAGTTACATCGACGTCAGTAAATGCTGAGCTGGGAGTTTAAGGAAACTG
CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG

>HF2_UF_09
ACAGGTTTGCATGGCTGTCGTCAGCTCGTGCAGTCTAGTTGGTGGTTAAGTCCCGCAACG
AGCGCAACCTCATCCTTAGTTACATCGACGTCAGTAAATGCTGAGCTGGGAGTTTAAGGAAACTG
CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG

>HF3_UF_09
GTGTTGCATGGCTGTCGTCAGCTCGTGCAGTCTAGTTGGTGGTTAAGTCCCGCAACG
AGCGCAACCTCATCCTTAGTTACATCGACGTCAGTAAATGCTGAGCTGGGAGTTTAAGGAAACTG
CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG

>PF4_CIT_09
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AGCGCAACCTCATCCTTAGTTACATCGACGTCAGTAAATGCTGAGCTGGGAGTTTAAGGAAACTG
CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG

>PF5_CIT_09
TGTTGCATGGCTGTCGTCAGCTCGTGCAGTCTAGTTGGTGGTTAAGTCCCGCAACG
AGCGCAACCTCATCCTTAGTTACATCGACGTCAGTAAATGCTGAGCTGGGAGTTTAAGGAAACTG
CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG

>PF6_CIT_09
TGTTGCATGGCTGTCGTCAGCTCGTGCAGTCTAGTTGGTGGTTAAGTCCCGCAACG
AGCGCAACCTCATCCTTAGTTACATCGACGTCAGTAAATGCTGAGCTGGGAGTTTAAGGAAACTG
CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG

>PF5_CIT_09
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AGCGCAACCTCATCCTTAGTTACATCGACGTCAGTAAATGCTGAGCTGGGAGTTTAAGGAAACTG
CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
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>PF6_CIT_09
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CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG

>PF6_CIT_09
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CCAGTGATAAACTGGAGGAAGGTGCGGGATATGTATTCAAGTCATCGTCCGCTTATG
AGTGGGACTACACATACGCTACAATGGTGGCTACAATGGGCTGCAAGTGCGAGGC
TAAGCCAATCCCTTTTTAGGCTGGATCTGCACTCGAATACGTGGAATACGGCCCTG
CAATGGTGCTACAAATGGGCTGCAAATGGGCTAAGCCAATCCCTTAAAGGC
CATCTCAAGTGCCAGGATATGCTCTGCAACCTGGAATGCTAGTA
ACGTGGATCAGCACGCCACGGTGAATACGTTCTCGGGTCTTGTACACTAGCTG
CACGCCCATGGGAATTGGTTTCACTCGAAGCTA
>PP23_CIT_09
GTCAAGCTGTTGCTGGAAGATGTGGTTGAAGTCCCGCAACGAGCGCAAC
CCTCATCTTTAGTTACATCAGTAATGCTGGGACTTTAAGGAAACTGCAAGTGT
AAACTGTTGAGGAAGTGTTGAGTGCATGTCATCTGCGGCTTATGGAGTGT
ACACACGTGCTACAAATGGTGCTACCATGCAAGTCGACTTCGGAATAGGCAA
CTCTTTAAAGGCCATCTCAGTGATATTCTATCTCGCAACTCGAATAGGGAAGCT
GAATGCTAGTAAATGTTGACTACGACAGCCTGAGGTGAATACGTTTCCGGGTCTTTGTA
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213
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A2. *Wolbachia* specific 16S rRNA nucleotide sequences used for phylogenetic reconstruction of *Frankliniella fusca* and *Thripinema fuscum* populations. Supergroup designation is listed after the host name of each *Wolbachia* strain.

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226
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A3. Nucleotide sequence and amino acid profile for Wolbachia surface coat protein (wsp) non-parasitized Frankliniella fusca cohort populations. Italicized nucleotides represent the forward primer.

Sample #11
> wsp
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Sample #20
> wsp
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I T P Y L G V G V A A Y I S N P S K A D A V K D Q K G F G F A Y Q A

Sample #7
> wsp
GAAATTTTTACCTTTTTATACAAAAGTGTGATGTTATTACACTATACGCA:GGA:AA
GGTAAAGGACAGCTCACTTAACAAAGATCTTTTTAGCTGGGGTGGGGCCATT TG
GTATAATAATGGTACATATTAGGATGTGTTAAGGCGTTACCTCAAACTTTGG
CTAAGATACAGCTGTAGTAATACTCTCTGAAACAAATGTTGCAAGACAGTATTAA
CAGCTTTTTACGATTGGTTAACCCTTTATTACGATATACGCACTAACAGGAA

231
Sample #8
>wsp
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AGCATTGTTACAGGATGTTACCAAGCTTTATTACGATATAGCAGATATGAC
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AAGCTGGTGTTAGCTATGATGTAACTCCAGAAATCAAACTCTTTGCTGGAGCTC
GTTACTTCGTTCTTTATGGTGCTAGTTTTGATAAGGCAACTAAGGATGATAATGG
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Sample #2
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CAAAAGCTGATGCAGTTAAAGATCAAAAAGGATTTGGTTTTGCTTATCAAGCAAA
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GTATCAAAAATGTTTACAGCGCTATTGTTGTGCAG

Sample #6
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AGCATTGTTACAGGATGTTACCAAGCTTTATTACGATATAGCAGATATGAC
TATACCTTTACCTTGGTGGTGGTGGTGGTGGTGCAGCATATATCAGCAATCTT
CAAAAGCTGATGCAGTTAAAGATCAAAAAGGATTTGGTTTTGCTTATCAAGCAAA
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>wsp
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TATCAAAAATGTGGTTTACACGGCTATTGGGTGCAG

EILFYTKVDGITICTGKEKDSPLSTRSFIAGGGAFGYKMDDIRVDVEGLYSQ
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VGAAAYISHPSKADAVKDQKGFGAYQAKAGVSYDVTPEIKLFAGARYFGSYG
ASFKATKDDNGIKNVVYSAIGA
A4. Nucleotide sequences of six non-parasitized *Frankliniella fusca* cohort populations at the provided loci for the MLST. Italicized nucleotides represent the forward primer and underlined nucleotides represent regions of polymorphism.

**Sample #11**

>gatB

GAAGCTGCAAGATGCAATGAAAAATTCAGCCAGATTTTGCGTTATATTGGTCTCA
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>fbpA

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Sample #8
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GAGATGGGTAAAGCAATGATTGACTGGAGAGGCAGAGGAGAAGATAGGG
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Sample #2
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AATTGTCTATACCGAGTAACTTTTGAAGATTTTTAATGGAAGTTTTAATGGAAT
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Sample #6
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GAGATGGGTAAAGCAATGATTGCTAAGAGGCAGAAGGAGAAGCATAGG
CAATTAGTTCGTCAGAGGCTGCGAGATTCCATTCTTGCTGAT
APPENDIX B
RECIPE FOR ELECTRON MICROSCOPY

2.5% Glutaraldehyde Fixative
10 ml 8% Glutaraldehyde
16 ml 0.2 M Cacodylate buffer
32 mg CaCl₂

1% Osmium Tetroxide Fixative
1 ml 4% Osmium tetroxide
1 ml 0.3 M Sucrose
2 ml 0.2 M Cacodylate buffer
Wrap in foil

Epon Araldite Plastic (in order)
4.5 gm DDSA
2 gm 812
1 gm 502
Warm to 60°C and mix well. Add 4 drops of activator after cool and Z-60-40 (100 µl/10 ml)

0.2 M Cacodylate Buffer
50 ml of Cacodylate buffer stock
6 ml of 0.2 M HCl
Double distilled water to make 100 ml

0.1 M Cacodylate buffer/Sucrose
5 ml 0.2 M Cacodylate buffer
5 ml double distilled water
0.1 gm sucrose

0.4 M Cacodylate buffer
42.8 gm of Cacodylate acid (sodium salt) and double distilled water to make 500 ml

Sucrose
5.1 gm sucrose
50 ml double distilled water

4% Osmium tetroxide
1 gm Osmium tetroxide
25 ml double distilled water
Wrap in foil; keep dark; store at 4°C

0.2 M HCl
1.6 ml of HCL
Double distilled water to make 100 ml
C1. RNA extraction protocol for Frankliniella fusca cohorts (modified from Sigma Technical Bulletin for TRI-Reagent).

1. Homogenize tissue samples in TRI-Reagent (20 thrips/250 µl).
2. Centrifuge homogenate at 12,000 × g for 10 minutes at 4°C to remove insoluble material.
3. Transfer supernatant to a fresh tube and allow samples to stand for 5 minutes at room temperature (RT).
4. Add 100 µl of chloroform, vortex for 15 seconds, and allow samples to stand for 15 minutes at RT.
5. Centrifuge at 10,000 × g for 15 minutes.
6. Transfer upper aqueous phase to a fresh tube and add 250 µl of isopropanol, invert gently, and allow samples to stand for 10 minutes at RT.
7. Centrifuge tubes at 16,000 × g for 10 minutes at 4°C.
8. Remove supernatant and wash the RNA pellet with 0.5 ml of 75% ethanol.
9. Centrifuge sample at 10,000 × g for 5 minutes and remove supernatant.
10. Dry the pellet for 10 minutes at room temperature.
11. Resuspend RNA in 20 µl of nuclease-free water and incubate at 37°C for 5 minutes.
C2. Primer sequence information for PCR.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Expected product size (in bp)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSWV N-protein</td>
<td>123</td>
<td>F(5’-CATTAGGATTGCTGGAGCTGAG-3’) R(5’-GACACCAGAGAAGCCCTAGGAA-3’)</td>
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<tr>
<td>28S</td>
<td>124</td>
<td>F(5’-GACCCGAAGAAGATGGAATGTGAACTATG-3’) R(5’-CGATTTAGCTTTCTGCCCCCTATAC-3’)</td>
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<tr>
<td>COI</td>
<td>143</td>
<td>F(5’-GTCGATTCTCGGAGCTAACC-3’) R(5’-CCGCTAGAAGATGGAGAGATA-3’)</td>
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<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (in μl)</th>
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<tbody>
<tr>
<td>Nuclease-free water</td>
<td>11.6</td>
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<tr>
<td>AMV/Tfl 5X reaction buffer</td>
<td>4</td>
</tr>
<tr>
<td>dNTP mix (10 mM)</td>
<td>0.4</td>
</tr>
<tr>
<td>Forward primer (100 μM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse primer (100 μM)</td>
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</tr>
<tr>
<td>MgSO₄ (25 mM)</td>
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<tr>
<td>AMV reverse transcriptase (5u/μl)</td>
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<tr>
<td>TFl DNA polymerase (5u/ μl)</td>
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<tr>
<td>RNA template</td>
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C3b. PCR thermal cycling profile used for one-step RT-PCR (modified from Promega Access RT-PCR System Technical Bulletin TB220).

<table>
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<td>45°C</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>1</td>
<td>15 minutes</td>
<td>70°C</td>
<td>AMV RT inactivation</td>
</tr>
<tr>
<td>1</td>
<td>3 minutes</td>
<td>94°C</td>
<td>cDNA/primer denaturation, polymerase activation</td>
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<tr>
<td></td>
<td>1 minute</td>
<td>94°C</td>
<td>Denaturation</td>
</tr>
<tr>
<td>1</td>
<td>1 minute</td>
<td>55°C</td>
<td>Annealing</td>
</tr>
<tr>
<td>40</td>
<td>1 minute</td>
<td>55°C</td>
<td></td>
</tr>
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<td></td>
<td>2 minutes</td>
<td>72°C</td>
<td>Extension</td>
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<tr>
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<td>7 minutes</td>
<td>72°C</td>
<td>Final extension</td>
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<td>∞</td>
<td>4°C</td>
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<table>
<thead>
<tr>
<th>Components</th>
<th>Volume (in µl)</th>
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</thead>
<tbody>
<tr>
<td>5X iScript reaction mix</td>
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<td>iScript reverse transcriptase</td>
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<tr>
<td>RNA template</td>
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C4b. PCR thermal cycling profile used for cDNA synthesis reactions (from iScript cDNA Synthesis Kit).

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<th>Temperature</th>
<th>Step</th>
</tr>
</thead>
<tbody>
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<td>30 minutes</td>
<td>42°C</td>
<td>Synthesis</td>
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<tr>
<td>1</td>
<td>5 minutes</td>
<td>85°C</td>
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<td>1</td>
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C5a. Recipe for a 20-µl volume reaction for quantitative real-time PCR (qPCR) (modified from Quantace SensiMixPlus SYBR & Fluorescein Kit).

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<tbody>
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<tr>
<td>Forward primer</td>
<td>1</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>1</td>
</tr>
<tr>
<td>Template</td>
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</tr>
<tr>
<td>Nanopure water</td>
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C5b. Thermal cycling profile used for quantitative real-time PCR (qPCR) (modified from Quantace SensiMixPlus SYBR & Fluorescein Kit).

<table>
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<tr>
<th>Cycle</th>
<th>Time</th>
<th>Temperature</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10 minutes</td>
<td>95°C</td>
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<td>40</td>
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<td>Denaturing</td>
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<tr>
<td></td>
<td>30 seconds</td>
<td>55°C</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>30 seconds</td>
<td>72°C</td>
<td>Extension</td>
</tr>
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C6a. MIDI analysis results for phenotype A.

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<th>Percent</th>
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Percent Named: 99.88% Total Amount: 302646

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C6b. MIDI analysis results for phenotype B.

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(Enterobacter)
C7. Sequencing of TSWV amplicon revealed 100% homology to *Tomato spotted wilt virus* isolate T992 nucleocapsid protein gene (Accession number AY848922).

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GCTGAGTATAGCAGCATACTCTTTCCCTTTCTTACCTGATTTACTTTTCAATAGCAAT
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CAGATTCTGATCTTCCTCAACACTCAAGGTCTTTGTGCTGATTCAAAAGCAACAAAT
GCTTTCTTAGTGAGCTTAAACCTTAGACATGATG
C8a. Standard Curve Titration of *Tomato spotted wilt virus* nucleocapsid gene (TSWV-N) for quantitative real-time PCR.

An external standard was prepared from a plasmid containing the TSWV-N gene (pBS-N2C4.5, see C8b for nucleotide sequence information) constructed by Kim *et al.* (1994) and sent by Anna Whitfield (Kansas State University). A map of the plasmid construct is provided below (Appendix C8c). To prepare the standard, purified plasmid DNA was transformed with the DH5-alpha strain of *E. coli* and digested with the restriction endonuclease BAM HI (Promega Corp., Madison, WI). The 40 µl reaction included 21.6 µl of water, 4.0 µ 10X RE of buffer, 0.4 µl of acetylated BSA, 10.0 µl of plasmid DNA and 4.0 µl of REN BAM HI. Contents were inoculated overnight at 37°C, and 4 µl of product was run on a 0.7% agarose gel at 90 V for 1 hour (Appendix C8d). Digestion resulted in a product of 4.5 kb in size. The digested product was purified (QIAquick PCR purification kit, Qiagen Sciences, MD) and the concentration of plasmid determined with a spectrophotometer (Thermo Scientific Nanodrop 1000 v3.7.1) to be 39 ng/µl. Based on the genome size (4,500 bp), the mass per copy was estimated to be $4.93 \times 10^{-18}$ g using the formula $m=n*1.096*(10^{-21})$, where $m =$ mass and $n=$size of genome. The mass (g) of plasmid DNA needed was determined by multiplying the desired copy number by the mass of haploid genome plasmid (g). Serial dilutions were made using the equation $C_1V_1= C_2V_2$, where $C_1=$ initial concentration (g/µl), $V_1=$volume of plasmid DNA, $V_2=$final volume (µl), and $C_2=$final concentration (g/µl).
C8b. Nucleotide sequence for the *Tomato spotted wilt virus* mRNA for the nucleocapsid (TSWV-N) protein (accession number X61799.1).

ATGTCTAAGGTTAAGCTCACTAAGGAAAGCATTGTTGCTTTGTTGACACAAGGCAAAGACCTTGAGTTTG
AGGAAGATCGAATCTGGTAGCATTCAACTTCAAGACTTTTTGTCTGGAAAACCTTGACCAGATCAAAAA
GATGAGCATTATTCATGTCTGACATTTCAACTTCAAGACTTTTTGTCTGGAAAACCTTGACCAGATCAAAAA
GATGAGCATTATTCATGTCTGACATTTCAACTTCAAGACTTTTTGTCTGGAAAACCTTGACCAGATCAAAAA
GATGAGCATTATTCATGTCTGACATTTCAACTTCAAGACTTTTTGTCTGGAAAACCTTGACCAGATCAAAAA

C8c. Map construct of the pBluescript plasmid used in generating the standard curve for quantitative real-time PCR.
C8d. Plasmid digestion gel. Lane 1 = 5 µl of a 1 kb ladder, lane 2 = undigested plasmid, and lane 3 = plasmid digested by BAMHI. Note the multiple plasmids joined together at the digestion site in lane 3 (arrows).
C8e. Serial dilution calculations used for generating the *Tomato spotted wilt virus* standard curve for quantitative real-time PCR.

<table>
<thead>
<tr>
<th>Dilution #</th>
<th>Source of plasmid DNA for dilution</th>
<th>Initial concentration (g/µl)</th>
<th>Volume of plasmid DNA (µl)</th>
<th>Volume of water (µl)</th>
<th>Final volume (µl)</th>
<th>Final concentration (g/ µl)</th>
<th>Resulting copy # of plasmid (per µl)</th>
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<tbody>
<tr>
<td>1</td>
<td>Stock (39 ng/ µl)</td>
<td>3.90 x 10^−8</td>
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<tr>
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<td>Dilution 2</td>
<td>4.93 x 10^−9</td>
<td>2.5</td>
<td>22.5</td>
<td>25</td>
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<td>1.0 x 10⁸</td>
</tr>
<tr>
<td>3</td>
<td>Dilution 3</td>
<td>4.93 x 10^−10</td>
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<td>22.5</td>
<td>25</td>
<td>4.93 x 10^−11</td>
<td>1.0 x 10⁷</td>
</tr>
<tr>
<td>4</td>
<td>Dilution 4</td>
<td>4.93 x 10^−11</td>
<td>2.5</td>
<td>22.5</td>
<td>25</td>
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<td>1.0 x 10⁶</td>
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<tr>
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<td>Dilution 5</td>
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<td>1.0 x 10⁵</td>
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BIOGRAPHICAL SKETCH

Kelly Renee Sims was born in 1979 in Royal Oak, Michigan. She is a graduate of Troy High School in Troy, Michigan. Kelly obtained a Bachelor of Science degree in resource ecology and management from the University of Michigan’s Department of Natural Resources and Environment in 2001. Kelly pursued her education and in 2003 graduated with a master’s degree from the University of Florida’s Department of Entomology and Nematology under the guidance of Dr. Joseph Funderburk. Kelly was awarded with an Alumni Fellowship, the highest graduate student award available, to pursue a Doctor of Philosophy at the University of Florida. In 2004, she continued her research project under the guidance of Dr. Joseph Funderburk, Dr. Drion Boucias, Dr. Stuart Reitz, Dr. James Becnel, and Dr. Timur Momol. She has received numerous scholarships and awards, and was a recipient of a National Science Foundation Science Partners in Inquiry-based Collaborative Education grant. Kelly is currently continuing her career as an entomologist with her husband in Atlanta, Georgia.