EFFECTS OF HELICOSPORIDIA ON MOSQUITOES

By

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The Helicosporidia are a poorly understood group of pathogens that have been detected worldwide in diverse groups of invertebrates. Discovered in 1921, their taxonomic position was debated until 2002, when it was determined that Helicosporidia are algae. Related to the vertebrate pathogen *Prototheca wickerhamii* Tubaki & Soneda, *Helicosporidium* represents the first described algal genus of invertebrate pathogens. The Helicosporidia are unique in that they produce a characteristic cyst stage composed of one elongate filamentous cell wrapped around three ovoid cells within a pellicle. On ingestion by a host, the cyst dehisces, releasing the filamentous cell and ovoid cells from the pellicle. The filamentous cell penetrates the midgut epithelium of the host and differentiates into the vegetative cell phenotype. Vegetative stages develop in the hemocoel via autosporulation, and eventually form cysts.

Recently, a new isolate of *Helicosporidium* was discovered in Florida that infects the blackfly *Simulium jonesi* Stone & Snoddy. Bioassays were conducted with this blackfly isolate (SjHe) against *Aedes aegypti* L. and *Anopheles quadrimaculatus* (Say).

Both species were infected at 7 days post-exposure. However, exposure to SjHe caused melanization and high early mortality in both species. There was also high variation in infectivity and mortality in all bioassays. In vitro dehiscence rates varied from one cyst preparation to the next, and may account for the high levels of variation. *Aedes aegypti* was selected for further bioassays due to low control mortality and synchronous development in this species. In *Ae. aegypti*, susceptibility decreased significantly after the first instar and also within the first instar with age. Most infected individuals died as fourth instar larvae; thus infected pupae and adults were rare. Infection did not significantly increase in *Ae. aegypti* maintained up to 3 weeks post-exposure. Susceptibility also decreased with increasing food availability during exposure. Food acted as a diluent, reducing the number of cysts ingested. Time to pupation was not significantly delayed in infected, individually reared *Ae. aegypti*. The presence of melanization, high initial mortality, and the high dosages necessary to achieve infection indicated a non-host interaction. This interaction was unexpected and contradicts the widely-held assumption that the Helicosporidia are not host-specific.

CHAPTER 1
INTRODUCTION

Since the first description by Keilin (1921), invertebrate pathogens in the genus *Helicosporidium* have been detected worldwide in diverse groups of invertebrates, including several orders of insects, mites, crustaceans, oligochaete worms, and trematodes (Sayre and Clark 1978; Purrini 1984; Avery and Undeen 1987b; Pekkarinen 1993). Until recently, their taxonomic position remained unclear. Keilin (1921) tentatively described *Helicosporidium parasiticum* as a protozoan, but Weiser (1970) proposed that the Helicosporidia were best placed among the lower fungi. Most recently, Boucias et al. (2001) suggested that the vegetative development of Helicosporidia was similar to the autosporogenic growth of unicellular algae. Significantly, genetic analysis defined the genus *Helicosporidium* as a member of the green algal class Trebouxiophyceae (Chlorophyta) and, as such, it represents a novel clade of invertebrate pathogens (Boucias et al. 2001; Tartar et al. 2002, 2003; Tartar 2004; Tartar and Boucias 2004; de Koning and Keeling 2004). The trebouxiophyte green algae are generally photosynthetic and free-living. However, the closest relatives to *Helicosporidium* (the genus *Prototheca*) are achlorophyllous algae that have evolved a heterotrophic life style, opportunistically infecting vertebrates.

The infectious cyst, the stage that defines the genus *Helicosporidium*, comprises three ovoid cells and a coiled, elongate, filamentous cell enclosed in an outer pellicle. The cyst dehisces when ingested by the insect host—the pellicle ruptures, releasing the filamentous cell and the three ovoid cells. The invasive filamentous cells pass through the

midgut epithelial layer and gain ingress to the hemocoel. Within the hemocoel, the filamentous cells differentiate into vegetative cells, which undergo autosporogenic cell divisions (Bläske-Lietze et al. in press). Vegetative cells have been observed to replicate within the phagocytic hemocytes and to develop extracellularly in the hemolymph (Bläske-Lietze and Boucias 2005). After multiple, 2- to 8-cell autosporogenic cell divisions, a portion of the hemolymph-borne vegetative cells differentiates into cysts (Bläske-Lietze et al. in press).

Boucias et al. (2001) reported on a *Helicosporidium* sp. isolated from a simuliid (*Simulium jonesi* Stone & Snoddy) by J. Becnel at United States Department of Agriculture Agricultural Research Service (USDA ARS) in Gainesville, Florida. This represents the fourth non-culicid nematoceran host record for *Helicosporidium*. Five species of mosquitoes have been found to be naturally infected by *Helicosporidium*. However, of the five recorded culicid isolates, only three have been examined in detail. Fukuda et al. (1976) found that a *Helicosporidium* sp. from *Culex nigripalpus* Theobald was infectious to 17 species of mosquitoes and 6 species of insects. Hembree (1979, 1981) discovered Helicosporidia infecting *Aedes aegypti* (L.) and *Culex quinquefasciatus* Say in Thailand and later evaluated the infectivity and biological control potential of this isolate. Seif and Rifaat (2001) performed a similar study with an isolate from *Culex pipiens* L. from Egypt. Bioassays with mosquitoes have also been performed with other isolates. Fukuda (1976) also reported melanization in mosquitoes infected with a beetle isolate. Avery and Undeen (1987b) studied the effects of a pond water isolate on three species of mosquitoes, noting melanization and high initial mortality. The simuliid isolate (designated SjHe), the subject of our study, has been assayed against six species of

Diptera, three species of Lepidoptera, and a weevil (Boucias et al. 2001; Bläske-Lietze et al. in press; Conklin et al. 2005).

The objective of our study was to further characterize mosquito-*Helicosporidium* interactions; particularly, those involving early mortality and the effect of larval age and food availability on early mortality, total mortality, and infection. Early mortality (defined as death before the production of cysts) was common in mosquitoes exposed to SjHe and a pond water isolate of *Helicosporidium* (Avery and Undeen 1987b; Conklin et al. 2005). Their studies suggested that early mortality and melanization indicated that mosquitoes were unsuitable hosts for these two isolates. With other mosquito isolates, mortality occurred at the larval-pupal interface. The early mortality observed by Avery and Undeen (1987b) and Conklin et al. (2005) may have been due to septicemia facilitated by ingress of the filamentous cell and may be mitigated by larval age or food level during exposure. In fact, age-based resistance to infection has been reported in three mosquito isolates of *Helicosporidium* (Fukuda et al 1976; Hembree 1981; Seif and Rifaat 2001). The effect of food availability on susceptibility has never been addressed for *Helicosporidium*, but food levels may influence the number of cysts ingested. Also, the presence of plant chemicals in the diet may alter the viability of *Helicosporidium* in the gut.

Finally, we also addressed the effect of *Helicosporidium* infection on development. Developmental delay due to infection has been noted in lepidopterans (Bläske and Boucias 2004), and a weevil (Conklin et al. 2005) but has not been quantified for mosquitoes, which have a shorter larval period than these other hosts. Hembree (1981) stated that development of infected *Ae. aegypti* was delayed 1 to 2 days, but did not offer

any data on host development. Conklin et al. (2005) presented head capsule measurements of mosquitoes exposed to *Helicosporidium*, which indicated that development was affected *Ae. aegypti* but not in *An. quadrimaculatus*. Data gathered from our experiments provide much-needed insight into the effect of *Helicosporidium* on mosquitoes and early mortality in mosquitoes exposed to *Helicosporidium*.

CHAPTER 2
LITERATURE REVIEW

Helicosporidium History

Helicosporidium was discovered in 1921 by D. Keilin parasitizing larvae of a ceratopogonid, *Dasyhelea obscura* (Winn.). Keilin (1921) described this new pathogen in great detail using fixed smears and sections. His hypothesized life cycle of *Helicosporidium parasiticum* included dehiscence of the spore by the filament, invasion of the host by the ingested "sporozoites," vegetative replication in the host hemocoel, and reformation of cysts (which later released their filaments and sporozoites after successive drying and wetting of the dead insect).

Keilin (1921) admitted that the question of the systematic position of *Helicosporidium* was difficult, ruling out its easy classification into the Cnidiosporidia (Sporozoa), Haplosporidia, Serumsporidia, and Mycetozoa. Keilin (1921) compared the polar capsule of Microsporidia and the spiral filament of *Helicosporidium* (Figure 2-1), concluding that the similarities between these two groups were superficial. The Haplosporidia, he argued, have a plasmodium stage and cysts surrounding the spores, both of which never appear in *Helicosporidium*. Also, the spores of the Haplosporidia are unicellular, unlike the four heterogeneous cells found in *Helicosporidium*. In the Serumsporidia, Keilin (1921) noted similarities to a group of parasites infecting Crustacea, but considered the descriptions of these species to be incomplete, making it impossible to judge the relationship between the Serumsporidia and *Helicosporidium*. Keilin (1921) saw no similarities between *Helicosporidium* and the Mycetozoa (slime

molds). Unlike *Helicosporidium*, the Mycetozoa have plasmodium and flagellate stages and *Helicosporidium* has more complicated spores than the Mycetozoa. In Keilin's final evaluation of *Helicosporidium*, he stated that this pathogen was a new type of Protist that could be temporarily included in the Sporozoa. Later, *Helicosporidium* was provisionally placed in its own order (Helicosporidia) within the Cnidiosporidia (Kudo 1931).

In the 1970s, there was a resurgence of interest in Helicosporidia as new isolates were discovered in Lepidoptera, Coleoptera, and mosquitoes. Based on observations of Keilin's original materials and an infected Hepialid caterpillar from Argentina, Weiser (1968, 1970) suggested that *Helicosporidium* be moved from the Protozoa to the primitive Ascomycetes of the family Saccharomycetaceae and subfamily Nematosporidiae. He argued that the structure of the vegetative stages of *Helicosporidium* did not resemble any of the protozoa, but he noted that the conservation of cell shape after separation of the daughter cells in Helicosporidium was similar that of to plants. Weiser (1970) contended that the filament of *Helicosporidium* was homologous to the needle-shaped ascospores of *Monosporella unicuspidata*, also described by Keilin (1920) in *Dasyhelea obscura*. Weiser (1970) also noted that *Helicosporidium*, like a typical fungal pathogen, caused lysis of host tissues. In the Hepialid he examined, however, the infection was limited to a cuticular wound, and it did not appear to spread throughout the host as in Keilin's *Dasyhelea* larvae. Instead, tumor-like cysts with a fibrous, multilayered lining enclosed the helicosporidial cells. No cells were found free in the hemolymph, and many of the tumor-like cysts contained melanized cells. Weiser hypothesized that the caterpillar was opportunistically infected through a wound, while *Dasyhelea* larvae, bathed in tree wound fluid containing Helicosporidia, could be infected

along the entire body, resulting in systemic infection. The trans-cuticular infection route was also similar to that of most entomopathogenic fungi. Soon, the first bioassays with *Helicosporidium* were carried out (Kellen and Lindegren 1974), and the first electron micrographs of *Helicosporidium* were produced (Lindegren and Hoffman 1976). These studies showed that *Helicosporidium* could be readily transmitted *per os*, and that, unlike the majority of Ascomycetes, the vegetative stages of *Helicosporidium* underwent mitotic division in the nucleus and contained well-defined Golgi bodies. These characteristics aligned *Helicosporidium* more closely with the Protozoa. After these studies, *Helicosporidium* remained *incertae sedis* for more than 25 years.

Identification of *Helicosporidium* as an Insect Pathogenic Alga

The diagnostic feature of *Helicosporidium* is the cyst stage (Figure 2-1 A, B). The cyst is round to discoid, with the appearance of a ridged biscuit (Boucias et al 2001) measuring 4-5 μm in width. Cyst measurements differ considerably from one isolate to the next (Table A-1) and may be indicative of species differences. Avery and Undeen (1987b) found that the average size of the cyst of a pond water isolate changed significantly after passage through a heterologous host, ranging from from 5.58 ± 0.03 μm to 5.20 ± 0.07 μm after the first passage. The change in cyst size presumably indicated adaptation to a new host. The cyst is composed of four cells in a multilaminate pellicle. The pellicle is thicker in the lateral region, peripheral to the filamentous cell (75-93 nm), than it is in the dorsal/ventral regions opposite the ovoid cells (50-53 nm) (Bläske-Lietze et al. in press). Three ovoid cells are stacked together along the central axis of the cell. These ovoid cells, originally referred to as "sporozoites", each possess a peripheral nucleus enclosing a granular cytoplasm (Boucias et al. 2001). The filamentous cell is wound around the ovoid cells, making 3 to 4 turns.

Upon physical or chemical stimulation, the cyst pellicle ruptures in the thinner, dorsal/ventral region, releasing the filamentous cell and ovoid cells. The free filamentous cell is highly resistant, either retaining a spiral conformation or straightening into a needle-like shape tapered at both ends (Figure 2-1 C). The length of the filamentous cell varies with the isolate, measuring 37 - 65 μm in length by 1 μm in diameter (Table A-2). Filamentous cells are covered in short projections or barbs (340 ± 60 nm), oriented in one direction. Vegetative cells (2-4 μm) are non-motile and also have a resistant pellicle with a textured outer surface (Figure 2-1 D, E). During autosporulation, the daughter cells are formed within the pellicle of the mother cell. Each pellicle contains 1, 2, 4, or 8 daughter cells (Boucias et al. 2001). The vegetative cells are uninucleate with elongate mitochondria and well-developed Golgi bodies (Lindegren and Hoffman 1976; Bläske-Lietze et al. in press). Once the daughter cells are released, the shell-like empty pellicle persists (Figure 2-1 F, G) and is diagnostic of active vegetative replication (Boucias et al. 2001).

Helicosporidium shares some morphological characteristics with algae. Vegetative replication by autosporulation is prevalent in green microalgae of the Chlorophyta, including the achlorophyllous genus *Prototheca* (Bold and Wynne 1978). The *Prototheca* were initially described as new organisms with homology to both yeast-like fungi and green algae (Krüger 1894). Nadakavukaren and McCracken (1973) demonstrated the presence of double-membraned starch storage granules (diagnostic of a plastid structure) in *Prototheca zopfii* Krüger. While a plastid structure has not been localized in *Helicosporidium*, the presence of a degenerate plastid is supported by molecular analysis (Tartar and Boucias 2004; de Koning and Keeling 2004. Ultrastructural studies show that

both *Helicosporidium* and *Prototheca* contain well-developed Golgi bodies and elongate, peripheral mitochondria (Lindegren and Hoffman 1976; Nadakavukaren and McCracken 1973). Significantly, *Helicosporidium* produces a specialized, diagnostic cyst stage, which is not seen among the *Prototheca*. Another important difference between the two parasites is that *Prototheca* only infect vertebrate hosts, while *Helicosporidium* is restricted to invertebrates.

There is extensive molecular evidence for the classification of *Helicosporidium* as a green alga and the relationship between *Prototheca* and *Helicosporidium*. Five genomic sequences have been amplified and sequenced: 18S, 28S, ITS1-5.8S-ITS2, actin and β-tubulin. All trees constructed with these sequences placed *Helicosporidium* with the green algae of the class Trebouxiophyceae (Tartar et al. 2002; Tartar 2004). An EST library has also been constructed, revealing a high number of unique sequences with no homology to any known sequences. Using this EST library, 98% of ribosomal protein sequences supported the green algal origin of *Helicosporidium* (de Koning et al. 2005). The mitochondrial *cox3* gene has also been sequenced in *Helicosporidium*, again demonstrating homology to green algae (Tartar 2004). For example, a Blast N search of the *Helicosporidium cox3* open reading frame gave E values of 5e-25 for *Prototheca wickerhamii* Tubaki & Soneda and 4e-13 for *Nephroselmis olivacea* Stein. The 16S ribosomal DNA sequence has been obtained from a remnant plastid in *Helicosporidium*, with significant homology to that of the Protothecan plastid. The plastid genome of *Helicosporidium* has been further characterized by amplification of a series of highly conserved genes known as the str-cluster. The arrangement of genes in this cluster indicates that *Helicosporidium* is closely-related to *P. wickerhamii*, but is more derived

than its close relative, having a re-organized, highly reduced genome (Tartar et al. 2004). Finally, the complete plastid genome of *Helicosporidium* has been sequenced by de Koning and Keeling (in press), confirming the highly reduced structure of the genome. The plastid genome of *Helicosporidium* is highly structured, only 37.5 kb, with very little non-coding DNA, no inverted repeats, encoding only the minimal number of tRNAs necessary.

There is also evidence that the plastid genome of *Helicosporidium* is functional. Several cDNA sequences for plastid-targeted genes have been identified in the EST library, suggesting that the plastid has retained functions for fatty acid metabolism, tetrapyrrole, isoprenoid, and amino acid biosynthesis. The plastid also appears to have a high reducing potential due to the presence of ferredoxin (de Koning and Keeling 2004). Many of these roles are shared by the apicoplast of *Plasmodium*, but the metabolic diversity of the *Helicosporidium* plastid exceeds that of *Plasmodium*. The evolution of the functional plastid of *Helicosporidium* may be the result of necessity, as the cyst must subsist in the environment before it is ingested by a host, or the plastid's metabolic diversity may be a relic of a more recent autotrophic ancestor (de Koning and Keeling 2004). Recently, Borza et al. (2005) characterized the plastid-targeted proteins in *P. wickerhamii*, revealing an even greater metabolic diversity than *Helicosporidium*, including carbohydrate metabolism and purine biosynthesis. This finding supports the hypothesis that plastid reduction is continuous along a parasitism gradient from the opportunistic parasites such as *P. wickerhamii* to the obligate parasites like *Plasmodium* spp. *Helicosporidium* appears to lie between these two extremes, as an obligate parasite

with a free infectious stage with the possibility of a facultative cycle under certain conditions (Boucias et al. 2001) and a moderately reduced plastid.

Although molecular analyses indicate a relationship between *Helicosporidium* and algae, the exact relationship between *Helicosporidium*, *Prototheca* and other non-photosynthetic algae remains unclear. Recent phylogenetic analyses of the genus *Prototheca* place *Helicosporidium* with *P. wickerhamii* and *Auxenochlorella protothecoides* (Krüger), basal to other *Prototheca* species (*Prototheca moriformis* Krüger, *Prototheca stagnora* (Cooke), *Prototheca ulmea* Pore, *P. zopfii*) (Ueno et al. 2005).

Pathology

Infection Cycle

The Helicosporidia are unique in their biology. There is no known organism with a similar cyst stage composed of one long filamentous cell coiled around three ovoid cells within a pellicle. When this cyst is ingested, it dehisces, releasing the filamentous cell. Dehiscence is probably a result of a combination of protease activity, and pH. Many of the known host insects of *Helicosporidium* are herbivores with very basic gut pH levels (pH 9-12), and lepidopteran gut extracts induce dehiscence (Boucias et al. 2001). However, proteases alone do not induce dehiscence in vitro, except with pre-treatment with the membrane permeability enhancer dimethyl sulfoxide (Bläske-Lietze et al. in press). Mechanical pressure also induces dehiscence (Boucias et al. 2001). High gut pH may be involved in dehiscence. Since dehiscence is necessary to achieve infection, the cyst's ability to dehisce is a direct indication of viability.

After dehiscence, the filament and the three ovoid cells or sporoplasms are released into the host gut. Initially, the sporoplasms were thought to be infective, while the

filamentous cell was a unique means of opening the cyst pellicle (Keilin 1921). It is now apparent that the filament itself is the infective cell that passes the midgut epithelium to initiate infection. In fact, the sporoplasms deteriorate in the gut (Boucias et al. 2001; Bläske-Lietze and Boucias 2005). Hypothetically, the passage of the midgut epithelium is aided by anchoring barbs that have been observed on the surface of the filamentous cell under SEM (Boucias et al. 2001). However, orientation of the barbs in the gut of lepidopteran larvae appears to indicate otherwise. Barbs are observed pointing toward the gut lumen on the lumen side (Figure 2-2 A), but point away from the gut once through the basal membrane (Figure 2-2 B inset) (Bläske-Lietze and Boucias 2005). Convergent evolution has produced similar ingress mechanisms in fungi and microsporidia. The polar filament of microsporidia is adapted to inject the parasite's cytoplasm into a susceptible midgut cell (Bigliardi and Sacchi 2001). Oomycete *Haptoglossa erumpens* likewise makes use of a specialized "gun cell" to inject the cell protoplasm into cells of its nematode host (Glockling and Beakes 2002). In addition to these intracellular parasites, extracellular parasites are also known to have needle-like penetration mechanisms. For example, the pathogenic yeast *Metschinikowia bicuspidata* var. *australis*, closely related to *Monosporella unicuspidata* described by Keilin (1920), has a club-shaped ascus which liberates its two needle-shaped ascospores upon digestion with snail gut juice or mechanical pressure, penetrating the host gut wall (Lachance et al. 1976).

Once across the midgut, the filamentous cell divides and releases the first vegetative cells. This stage of infection is transitory, but the filamentous cell has been observed emerging from the basal side of the midgut 4-24 hours post-exposure (Figure 2-2 B). While these filaments appear to pass through the midgut completely, filamentous

cells are not observed to be freely circulating in the hemolymph. Instead, early vegetative stages appear in the hemolymph and hemocytes 48 hours post-exposure (Bläske-Lietze and Boucias 2005). The filamentous cell pellicle can be observed inside hemocytes with early vegetative stages (Figure 2-3). Thus, the host hemocytes appear to be the site of initial replication. The process of filament to vegetative cell transformation has also been observed by in vitro studies (Bläske-Lietze et al. in press). In vitro, within 24 hours, the cytoplasm and nucleus of the filament migrate to one end, causing a swelling and shortening of the filament. Nuclear division followed by cytoplasmic division produces 4 daughter cells within the pellicle of the filament. At 48 hours, the filamentous cell is observed to rupture along the horizontal axis of the pellicle, releasing the four elongate cells (Figure 2-2 C), which then divide into eight oval to spherical vegetative cells. These vegetative cells undergo autosporulation, colonizing the hemocoel of the host.

During autosporulation, nuclear division followed by cytokinesis, and formation of daughter cell pellicles all take place within the mother cell pellicle. *Helicosporidium* produces two, four, or eight daughter cells per mother cell. Secretion of new pellicles and the shedding of the mother pellicle can occur at two, four, and eight-cell stages, but also appears to occur often in single-celled vegetative stages, though the significance of shedding the pellicle at this stage is unknown (Bläske-Lietze et al. in press). Autosporulation has been observed to occur both intracellularly and in the hemocoel. Autosporulation continues for many cycles. In vitro cultures experience a lag phase of 1.5 days and a log growth phase between 1.5 and 6 days, reaching the stationary phase at 7 days with a density of 1.27×10^5 cells/μL. The doubling time of in vitro cultures during

the exponential phase is eight hours. Likewise, in vivo, the doubling time in the hemolymph is seven hours (Bläske-Lietze et al. in press).

Several days after infection, cysts begin to form in the host. The timing of cyst formation may be related to the development of the host, as cyst formation has been observed to occur more slowly in long-lived hosts (Conklin et al. 2005). The stimulus to produce cysts in the host is unknown. Cyst formation does not occur in vitro, but injection of in vitro produced vegetative cells consistently results in cyst formation. High cell densities and media depletion do not appear to be important in cyst differentiation. The death of the host insect also does not appear to affect cyst differentiation. It is thought that the cyst is derived from the 4-cell vegetative stages which are produced at a genetically determined rate, since the proportion of cysts produced from in vitro cultures when injected into a host corresponds to the proportion of 4-cell vegetative stages present in the culture (Bläske-Lietze et al. in press). Rather than forming cysts, adherent, multicellular clusters of cells known as pamelloid colonies form during long-term in vitro culture (Figure 2-4). These colonies reflect an inability of the cells to separate during autosporulation, and have been observed in other green algae (Bläske-Lietze et al. in press). Once formed, presumably the cysts are released into the environment to infect the next generation of hosts. The method of cyst dispersal is still unknown. Cysts appear to be retained in the insect after death (Bläske-Lietze et al. in press). Vertical transmission is possible in lepidopteran hosts at a low frequency (Bläske and Boucias 2004). However, vertical transmission appears to be incidental, as Helicosporidia have not been observed invading ovaries (Bläske and Boucias 2004). Fukuda et al. (1976) found no vertical transmission in *Culex salinarius* Coquillett.

Host Response

The host response to *Helicosporidium* infection is variable. Gross signs of infection such as slow movement and severely retarded growth have been observed in a few hosts (Keilin 1921; Hembree 1979; Conklin et al. 2005). The primary sign of infection is milky white hemolymph (Boucias et al. 2001; Sayre and Clark 1979; Conklin et al. 2005). In lepidopteran hosts, infected larvae often molt into deformed pupae or adults (Bläske and Boucias 2004).

Delayed development appears to occur in some hosts (Hembree 1981; Bläske and Boucias 2004; Conklin et al. 2005). Hembree (1981) reported that infected *Ae. aegypti* larvae pupated 1-2 days later than uninfected larvae and that pupation was lengthened. Quantitative data on this observed delay in development was lacking, however. Bläske and Boucias (2004) found that developmental delay varied among susceptible noctuid species. Pupal weight was reduced in infected *H. zea*, but not in *Tricoplusia ni* (Hübner) or *Spodoptera exigua* (Hübner). Pupation was delayed in *H. zea* and *S. exigua*, but not in *T. ni*. Eclosion was delayed in *S. exigua*, but not in *H. zea* or *T. ni*. However, a high proportion of infected adults and pupae of all species were malformed and adult longevity of infected individuals was reduced for all species (Bläske and Boucias 2004). Conklin et al. (2005) reported reduction in larval weight gain in infected *Diaprepes abbreviatus* (L.).

Helicosporidium, for the most part, appears to be able to effectively evade internal host defenses. The filamentous cell is apparently attacked and phagocytosed during initial infection events, but resists lysis within the phagosome, producing daughter cells that are eventually released from the hemocytes (Bläske-Lietze and Boucias 2005). Boucias et al. (2001) found that circulating hemocytes did not attack vegetative cells. Unlike

entomopathogenic fungi that reduce the number of circulating hemocytes or impede phagocytosis, the hemocytes appear to be normal in both appearance and number in Helicosporidia-infected hosts (Bläske-Lietze et al. in press; Boucias et al. 2001). While the majority of *Helicosporidium*-host interactions have shown the remarkable stealth of *Helicosporidium*, there are four reports in the literature of encapsulation, nodulation, or melanization in response to infection. Weiser (1970) described the formation of multicellular sheaths surrounding *Helicosporidium* that appeared to originate from a cuticular wound. The cuticle had formed a callus over the wound, enclosing some vegetative stages and cysts. Around the wound, the masses of helicosporidial cells were encapsulated by layers of cells originating in the hypodermis with no hemocytes evident. Helicosporidial cells were melanized in most areas. In addition to the cells encapsulated near the cuticular wound, helicosporidial cells were found encapsulated by lymphocytes in other areas of the host. Likewise, Boucias et al. (2001) found that *Galleria mellonella* injected with purified cysts phagocytosed the cysts formed hemocytic granulomas, melanized hemocytes attached to various tissues. Within these granulomas, vegetative cells continued to multiply, and invaded host tissues, but no freely-circulating vegetative cells were observed. Fukuda et al. (1976) found that a *Helicosporidium* isolate from a beetle produced localized infections in mosquito larvae along with melanization, but a mosquito isolate tested in the same study produced systemic infections with no melanization. A pond-water isolate produced a similar response in mosquito larvae (Avery and Undeen 1987b, Kim and Avery 1986). It is apparent that the origin of the isolate may have an affect on whether or not the host will exhibit an immune response to vegetative replication.

Typically, infection by *Helicosporidium* does not cause acute mortality in insect hosts. Most infected insects live until pupation or even adulthood (Fukuda et al. 1976; Hembree 1981; Bläske and Boucias 2004; Bläske-Lietze and Boucias 2005; Bläske-Lietze et al. in press). The precise cause of death is unknown, though death at the larval-pupal or pupal-adult interface may indicate a disruption in molting behavior or endocrine system. At the time of death, the host hemocoel is full of helicosporidial cells. In *Helicoverpa zea*, 9.7×10^6 cells are present in each microliter of hemolymph at 10 days post-exposure. Such a large number of invasive cells may restrict hemolymph flow and deplete nutrients.

Early mortality has been reported in mosquito larvae. Fukuda et al. (1976) report that first instar *Cx. salinarius* exposed to high dosages for a long period of time rarely survived to the fourth instar, but did not mention the precise time at which this mortality occurred. Avery and Undeen (1987b) report that the majority of mortality to mosquito larvae occurred within 72 hours post-exposure, and attributed this response to septicemia.

Host Records and Ecology

Members of the genus *Helicosporidium* have been discovered worldwide, parasitizing a wide range of invertebrate taxa. *Helicosporidium parasiticum*, the only described species in the genus, was discovered in larvae of a ceratopogonid living in tree wounds in Cambridge, England. There are 49 reports of natural *Helicosporidium* infections in invertebrates, spanning 37 described species and five continents (Table 2-1). Natural infections have occurred in mites, cladocerans, Coleoptera, Collembola, Diptera, Lepidoptera, Oligochaeta, and parasitic trematodes. Most natural infections are associated with hosts that inhabit moist environments such as tree wounds (Keilin 1921; Yaman and Radek 2005), water (Avery and Undeen 1987a; Bläske and Boucias 2004; Boucias et al.

2001; Chapman 1967, 1974; Fukuda et al. 1976; Hembree 1979; Pekkarinen 1993; Sayre and Clark 1978; Seif and Rifaat 2001), rotten fruit (Lindegren and Okumura 1973), and forest soils (Purrini 1979, 1980, 1981). Unlike the *Prototheca*, which are often isolated as free vegetative cells in sewage, soil, and tree wounds, vegetative cells of Helicosporidia have never been isolated outside of a host.

In laboratory assays, various *Helicosporidium* isolates have been transmitted to 75 known species of mites, Coleoptera, Diptera, and Lepidoptera. (Table 2-2) Only one assay has been performed on a vertebrate system. *Helicosporidium* was intubated into the stomachs of 12 golden hamsters. Thirty days later the hamsters were examined and found free of infection (Hembree 1981). Significantly, *Helicosporidium* does not grow in vitro at 35ºC, and cysts have reduced infectivity when stored at 37ºC for more than a few hours (Boucias et al 2001; Hembree 1981).

Helicosporidium **and Mosquitoes**

Mosquitoes are one of the best-studied hosts of *Helicosporidium*. The discovery of *Helicosporidium* in a single larva of *Culex territans* Walker (Chapman 1967) first opened the question of the biological control potential of *Helicosporidium*. While *Helicosporidium* has been transmitted to 27 species in 8 genera of Culicidae in the lab, natural infections have only been found in 5 species of mosquito: 4 *Culex* spp. and *Ae. aegypti*. These isolates have originated from Lousiana, Thailand, and Egypt. Two other nematoceran host records exist. The first described host of *Helicosporidium* was a ceratopogonid (Keilin 1921), and *Helicosporidium* has also been isolated from *S. jonesi* in Florida.

Of the 27 species of mosquito infected in the laboratory, 25 of these species were infected by mosquito isolates, 7 by heterologous isolates, and 5 by both mosquito and heterologous isolates. Mosquito isolates have been transmitted to 6 species of Lepidoptera and Coleoptera. *Anopheles* spp. have been reported to be highly susceptible to both a mosquito isolate (Fukuda et al. 1976) and a heterologous isolate (Avery and Undeen 1987b). However, *Culex* spp. are also very susceptible to mosquito isolates (Fukuda et al. 1976; Hembree 1981; Seif and Rifaat 2001). Predatory *Toxorhynchites splendens* (Wiedemann) larvae are susceptible to *Helicosporidium* infection if fed infected larvae (Hembree 1981).

Bioassay systems have varied considerably from isolate to isolate, making comparisons between isolates difficult. The various bioassay methods for the major mosquito bioassays are summarized in Table 2-3. Low dosages and long exposure times probably best imitate natural infection conditions. Fukuda et al (1976) and Hembree (1981) do not specify at what time post-exposure infection was assayed. Seif and Rifaat (2001) reported that the time of assay was 10 days after exposure, while Avery and Undeen (1987b) and Kim and Avery (1986) assayed for infection at adult emergence. In most assays live vegetative stages or cysts indicated infection, but the infection criteria of Avery and Undeen (1987b), included live and melanized helicosporidial cells.

In all mosquito bioassays, *Helicosporidium* acted in a dosage-dependent manner. However, due to variations in bioassay methods, estimated IC_{50}'s varied greatly. For example, the estimated IC_{50} of *Cx. salinarius* was greater than 9.1×10^6 cysts/mL when measured by Fukuda et al. (1976), but was only 2.6×10^4 cysts/mL when measured by Avery and Undeen (1987b). Hembree (1981) and Seif and Rifaat (2001) had comparable

exposure methods, resulting in similar IC_{50}'s for the two *Aedes* sp. they tested. The estimated IC_{50}'s for each bioassay are summarized in Table 2-4. In addition to the concentration of cysts in the exposure container, the time of exposure was directly related to infection rates. Longer exposure led to higher levels of infection and mortality in all bioassays (Fukuda et al. 1976; Avery and Undeen 1987b; Seif and Rifaat 2001). The degree to which exposure time had an effect depended on the dosage tested and the age of the larvae. If 24-hour old larvae were exposed for 24 hours, they would often molt into the next instar during the exposure time, introducing another variable of physiological age (Hembree 1981). Hembree (1981) exposed 24-hour old *Ae. aegypti* to 5×10^2 cysts/mL for a series of times from 1 hour to 48 hours. At this dosage, there was a 3-fold increase in infection by increasing exposure time from 1 to 48 hours. Seif and Rifaat (2001), on the other hand, tested third instar *Cx. pipiens* at the same dosage, and showed a 10-fold increase in infection from 1 hour to 48 hours. Results at higher dosages (1×10^3 and 5×10^3 cysts/mL) were nearly identical for the two bioassays. Fukuda et al. (1976) reported a 9-fold decrease in number of surviving larvae from 1 hour to 8 hours, but the infection rate did not follow the same trend, remaining around 50% for exposure times of 1, 2, and 8 hours.

Susceptibility decreased rapidly with larval age. Hembree (1981) notes that physiological age (instar) rather than chronological age is the most important factor for susceptibility. In his assays with *Ae. aegypti* exposed to 1×10^3 cysts/mL, percent infection dropped from 63% to zero from 24-hour old larvae (first instar) to 48-hour old larvae (second instar). Likewise Fukuda et al. (1976) found a 3-fold reduction in infection rate in larvae 1 day to 3 days old. Seif and Rifaat (2001) reported that the IC_{50} of fourth

instar *Cx. pipiens* was 7-fold higher than the first instar larvae, and the second instar larvae just 1.2-fold higher than the first instar larvae.

Biocontrol Potential

In order for a biocontrol agent to be successfully integrated into an IPM strategy, it must be safe, easy to obtain, inexpensive, and able to survive storage and environmental extremes. *Helicosporidium* appears to be safe to vertebrates due to temperature limitations, though no systematic safety analysis has been performed on *Helicosporidium*. The wide host range of *Helicosporidium* may be problematic, however, in systems where non-target invertebrates are a concern. Feasibility of biological control has been assessed by examining in vivo and in vitro production of cysts, storage, and the effects of environmental conditions on cyst viability.

In Vivo Production

Helicosporidium has been successfully mass-produced in vivo in *Cx. salinarius* (Fukuda et al. 1976), *Ae. aegypti* (Hembree 1981), and *Cx. pipiens* (Seif and Rifaat 2001) as well as *S. exigua* (Hembree 1981), *Carpophilus mutilatus* (Erichson), *Paramyelois transitella* (Walker) (Kellen and Lindegrean 1973), *H. zea* (Avery and Undeen 1987b; Boucias et al. 2001; Bläske and Boucias 2004), and *Spodoptera littoralis* (Boisduval) (Seif and Rifaat 2001). Methods for in vivo production have changed over time. Mosquito hosts were invariably infected by exposure to a cyst suspension of a known concentration. Hembree (1981) reports optimization procedures to determine the appropriate dose, exposure time, and age of larvae to use to produce the highest number of cysts per insect. Lepidopteran hosts, on the other hand, were infected by several different methods. Kellen and Lindegren (1974) infected *P. transitella* larvae with diet containing 2.6×10^6 cysts/g, though the methods by which these cysts were obtained and

integrated into the diet are not described. Hembree (1981) injected *S. exigua* larvae with gradient-purified cyst preparations. Avery and Undeen (1987b) allowed starved *H. zea* larvae to feed for 24 hours on a droplet containing cysts. Seif and Rifaat (2001) fed *S. littoralis* on castor oil leaves treated with a droplet containing cysts. Purification protocols likewise vary. Infected mosquitoes were collected and homogenized in deionized water. In some studies, this was the extent of the cyst purification protocol (Fukuda et al. 1967; Hembree 1981). Seif and Rifaat (2001) added a centrifugation step into the purification protocol for *Cx. pipiens*. Lepidopteran hosts, having much more tissue and cellular debris, were macerated and filtered, then cysts were purified on Ludox or Percoll gradient centrifugation using methods similar to Undeen and Vavra (1998) for purification of microsporidia (Avery and Undeen 1987b; Boucias et al. 2001).

The number of cysts produced in different insect hosts is summarized in Table 2-6. Although cyst production in lepidopteran hosts has been reported to be 2-3 orders of magnitude higher than mosquito hosts, infectivity of cysts may be reduced by amplification in a heterologous host. For example, Hembree (1981) reported that cysts produced in *S. exigua* were less infectious to *Ae. aegypti* than cysts produced in *Ae. aegypti*. Seif and Rifaat (2001) also produced cysts in a lepidopteran host but did not compare the infectivity of lepidopteran-produced cysts with mosquito-produced cysts. Interestingly, Avery and Undeen (1987b) reported significant changes in cyst size after as little as one passage through a heterologous host, indicating a rapid shift in phenotype based on host (Avery and Undeen 1987b).

In Vitro Production

Helicosporidium is capable of growth on many kinds of media. Boucias et al. (2001) found that vegetative growth was possible on insect tissue culture medium,

Candida liquid broth, Vogel-Bonner minimal broth, and SD broth. The only medium tested that did not support vegetative growth was Czapek Dox broth, which only supports growth of organisms capable of using inorganic sources of nitrogen. Cysts placed in enriched growth media will dehisce, releasing filamentous cells which produce vegetative cells (Boucias et al. 2001). Vegetative replication continues for many cycles in vitro, eventually resulting in pamelloid colonies (Bläske-Lietze et al. in press). Cyst formation has never been observed in vitro. More research is needed to understand the cues requisite for cyst formation. If the signals can be determined, in vitro production of cysts may be possible in the future.

Storage and Stability

Storage of *Helicosporidium* has been addressed several times over, but it is difficult to compare many of these experiments due to differences in methods or time of storage. High temperatures consistently deactivate *Helicosporidium*. Hembree (1981) reported that *Helicosporidium* exposed to 42ºC for 24 hours has significantly lower infectivity, and exposure to 50ºC for even 15 minutes reduced transmission at the highest concentration to only 4%. However, cysts withstood 24ºC and 32ºC for 24 hours with no loss of infectivity. At room temperature, storage for 10 days reduced infectivity to only 28% at a dosage of 3×10^5 cysts/mL, and after 17 days of storage at room temperature, infectivity dropped to 6%. In vitro, vegetative stages do not replicate at 35ºC, and cells exposed to 35ºC for 4 days are killed (Boucias et al. 2001).

Cold storage tolerance varies among *Helicosporidium* isolates. In general, purified cysts retain infectivity well when stored in deionized water at 4-5ºC, an average household refrigerator temperature (Hembree 1981; Seif and Rifaat 2001; Avery and Undeen 1987b). Hembree (1981) reported that, when stored at 4-5ºC, cysts began to lose

infectivity around 6 weeks. Seif and Rifaat (2001), however, reported that storage at the same temperature preserved infectivity through 6 months. Avery and Undeen (1987b) reported that the LC_{50} of cysts stored for 3 months at 5ºC increased from 8.3×10^3 to 4.3×10^5 cysts/ml. At -15ºC, or household freezer temperature, Avery and Undeen (1987b) and Seif and Rifaat (2001) reported that infectivity was retained for 6 months. The data of Seif and Rifaat (2001) indicated that cysts frozen at -15ºC began to show decline quicker than the cysts stored at 5ºC. Hembree (1981) also reported that cysts frozen at -70ºC in cryoprotectant retained infectivity after 6 months of storage, and storage at this temperature without cryoprotectant for 6 months destroyed infectivity. It is possible that cryoprotectants at ultra-low temperature may be a long-term storage solution.

The effect of dessication on cyst viability is also variable. Hembree (1981) found that lyophilized and vacuum-dried cysts completely lost infectivity after 4 weeks at room temperature. Avery and Undeen (1987b) air-dried cysts at 5ºC, and found that the LC_{50} of dried cysts stored for 5 days increased from 8.3×10^3 cysts/mL to 9.2×10^4 cysts/mL, indicating a 10-fold loss of infectivity. Seif and Rifaat (2001) found that air-dried spores held at room temperature had lost all infectivity when assayed after 3 months of storage

Hembree (1981) performed a series of experiments evaluating the effect of environmental conditions on *Helicosporidium* infectivity. One hour exposure to UV light destroyed cyst viability. Exposure to buffer solutions with pH ranging from 10.5 to 3.0 for 24 hours at 4ºC had no significant affect on viability. A 5% solution of household detergent for 24 hours at 4ºC had no effect on infectivity, but a 10% solution of detergent reduced infectivity by 20-43%. Exposure to saline (NaCl) for 24 hours eliminated

viability at 1.71 M (10% NaCl by weight). At 0.85 M (5%), infectivity was reduced by 8 to 52%.

Table 2-1. Side-by-side comparison of the filamentous cell of Helicosporidium and the polar-capsule filament of Cnidosporidia (Microsporidia) modified from Keilin (1921). Later it would be shown that the filamentous cell does in fact unroll in the intestine of a host, demonstrating convergent evolution of these two pathogen ingress mechanisms.

Spiral filament of *Helicosporidium*	Polar-capsule filament of Cnidosporidia
(1) Filament is not enclosed in a polar capsule but lies free beneath the wall of spore.	(1) Filament is enclosed in a capsule of which it forms a part.
(2) Filament always unrolls in the dead body of its host.	(2) Filament does not unroll until spores reach intestine of a second host.
(3) Filament unrolls slowly.	(3) Filament is shot out.
(4) Filament is pointed at both ends and is wide and ribbon-like in the middle.	(4) Filament is pointed at only one end and very fine.
(5) Axial portion of filament is very chromatic, nucleus is well formed in anterior third of filament.	(5) No chromatic axial portion, degenerated nucleus upon wall of terminal capsule.
(6) Filament is robust and very resistant in all media.	(6) Filament fine and very fragile.

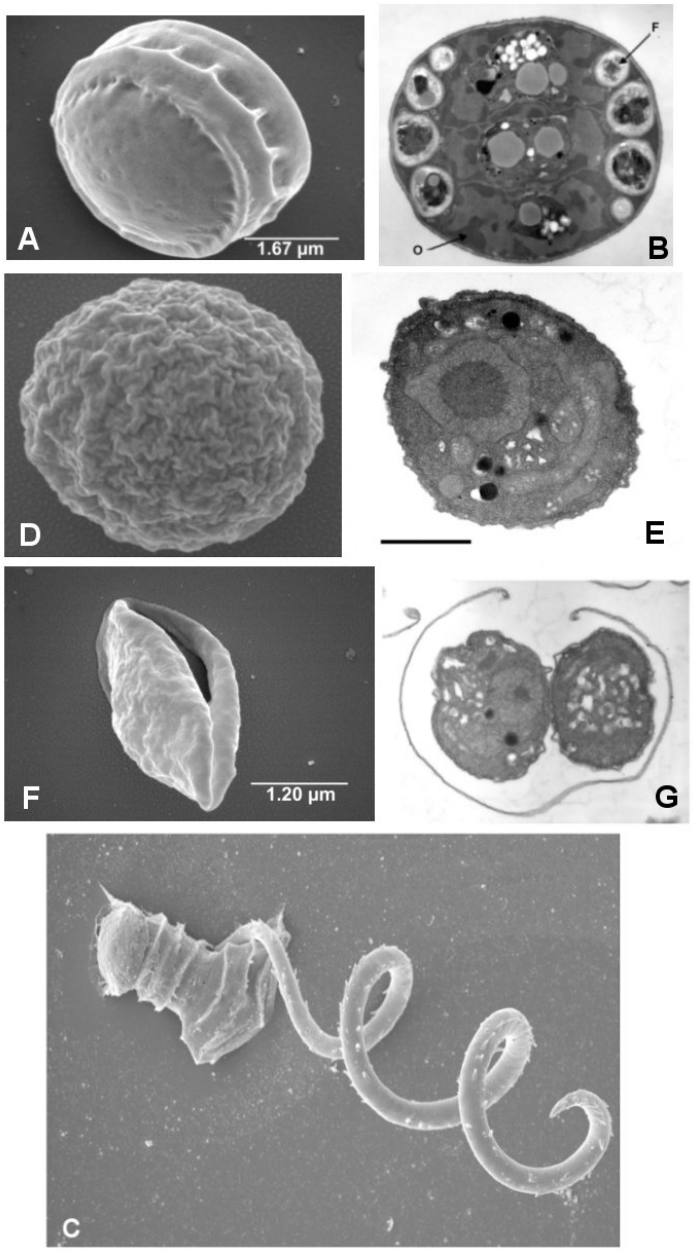

Figure 2-1. Cell types and structures of Helicosporidia. A) SEM image of the cyst. B) TEM image of the cyst. C) SEM image of a filamentous cell being released from a cyst: note barbs on surface. D) and E) SEM and TEM images of the vegetative cell. F) and G) SEM and TEM images of the vegetative pellicle. (Boucias et al. 2001; Bläske-Lietze et al. in press, used with permission)

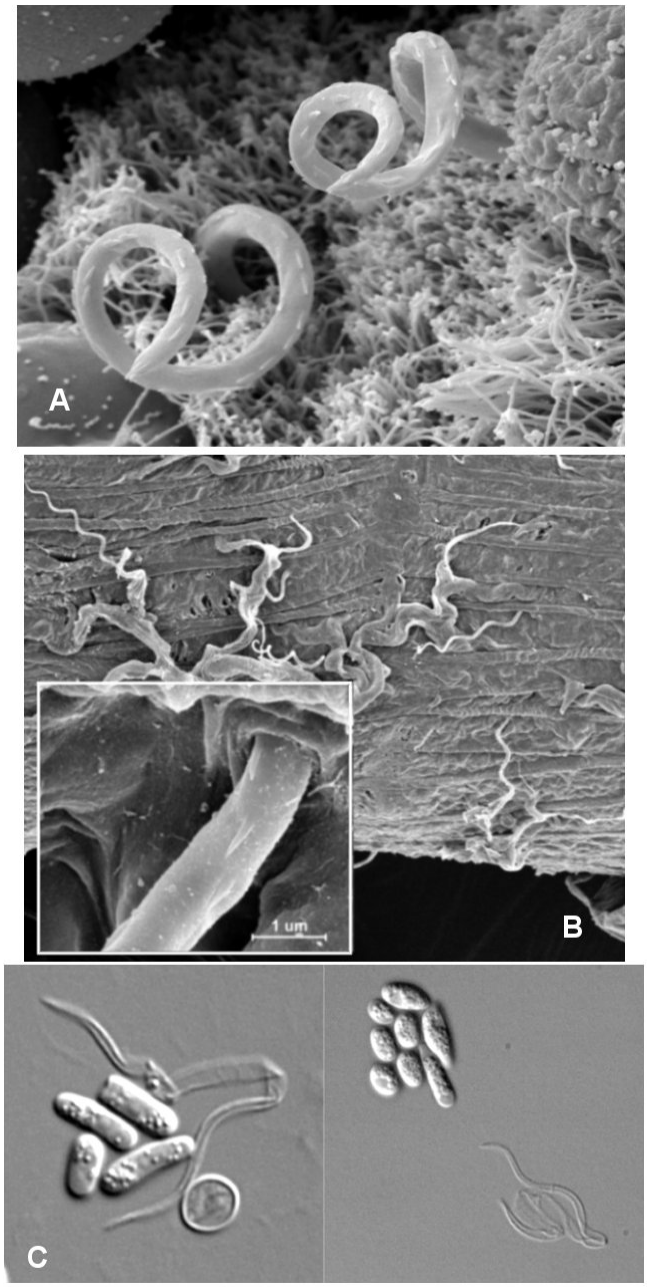

Figure 2-2. Initial infection events and development of the filamentous cell. A) Shows the filamentous cell embedded in the midgut microvilli, B) the filamentous cells emerging from the basal lamina of the midgut B inset: note orientation of barbs is away from gut lumen once through the basal lamina, C) the in vitro development of the filamentous cell, producing 4 elongate daughter cells which divide into 8 spherical vegetative cells. (Boucias et al. 2001; Bläske-Leitze and Boucias 2005; Bläske-Leitze et al. in press, used with permission)

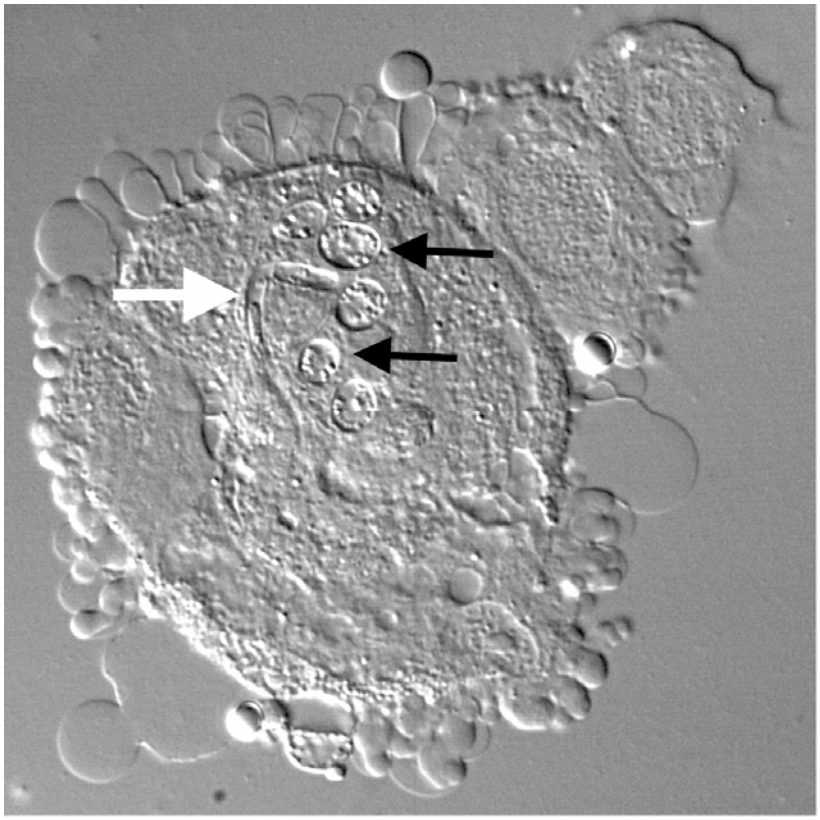

Figure 2-3. Filament development in vivo within a hemocyte. Arrows indicate early vegetative cells and remainder of filamentous cell pellicle. (Bläske-Leitze and Boucias 2005, used with permission)

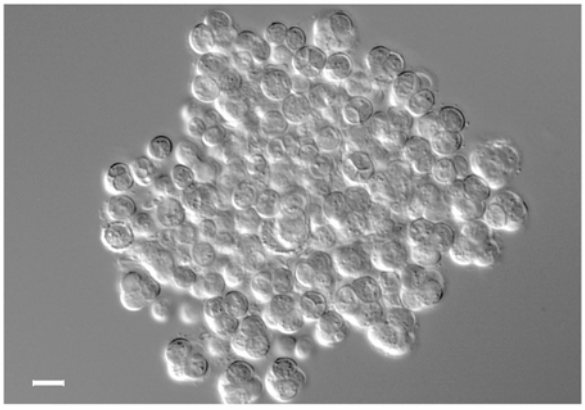

Figure 2-4. Pamelloid colony formed in vitro after several media transfers. (Bläske-Lietze et al. in press, used with permission)

Table 2-2. Natural hosts of *Helicosporidium*

Group	Species	Locations	References
Coleoptera	13	USA, Mexico, Hawaii, Germany, Africa, Turkey	Kellen and Lindegren 1973; Lindegren and Okmura 1973; Purrini 1980, 1985; Bläske and Boucias 2004; Yaman and Radek 2005
Acarina	11	England, Germany	Keilin 1921; Purrini 1981, 1984
Diptera	10	England, USA, Thailand, Germany, Egypt	Keilin 1921; Chapman 1967, 1974; Hembree 1979; Fukuda et al. 1976; Purrini 1980; Seif and Rifaat 2001; Boucias et al. 2001
Collembola	3*	Germany, USA	Purrini 1984; Avery and Undeen 1987a
Cladocera	1	USA	Sayre and Clark 1978
Lepidoptera	1	Argentina	Weiser 1970
Other (Oligochaeta, Trematoda)	2	Germany, Finland	Purrini 1980; Pekkarinen 1993

*2 identified species

Table 2-3. Laboratory-produced *Helicosporidium* infections

Group	Species	References
Acarina	3	Kellen and Lindegren 1973
Coleoptera	13	Kellen and Lindegren 1973; Fukuda et al. 1976; Conklin et al. 2005
Diptera	26	Kellen and Lindegren 1973; Sayre and Clark 1978; Hembree 1979, 1981; Fukuda et al. 1976, 1985; Kim and Avery 1986; Avery and Undeen 1987a,b; Seif and Rifaat 2001; Boucias et al. 2001
Lepidoptera	11	Kellen and Lindegren 1973; Fukuda et al. 1976; Kim and Avery 1986; Avery and Undeen 1987a,b; Hembree 1981; Seif and Rifaat 2001; Boucias et al. 2001; Bläske and Boucias 2004

Table 2-4. Summary of bioassay methods for four major mosquito studies

Authors	Isolate source	Exposure time (hours)	Dose (cysts/mL)	Volume (mL)	Number of larvae
Fukuda et al. 1976	*Cx. nigripalpus, Cx. salinarius*	1-8	2×10^5 to 1×10^7	5	500-1000
Hembree 1981	*Ae. aegypti*	24	5×10^2 to 5×10^4	20	100
Avery and Undeen 1987b	Pond water	24	1×10^3 to 4×10^5	100	150
Seif and Rifaat 2001	*Cx. pipiens*	24	5×10^2 to 8×10^3	100	100

Table 2-5. The IC_{50} of first instar larvae as recorded for various isolates and species.

Authors	Species	IC_{50} (cysts/mL)
Fukuda et al. 1976	*An. quadrimaculatus*	$\sim 1.7 \times 10^5$
	Cx. salinarius	$> 9.1 \times 10^6$
Hembree 1981	*Ae. aegypti*	$\sim 1 \times 10^3$
Avery and Undeen 1987b	*An. quadrimaculatus*	4.4×10^2
	Ae. aegypti	2.2×10^4
	Cx. salinarius	2.6×10^4
Seif and Rifaat 2001	*Cx. pipiens*	1.9×10^2
	Cx. atennatus	5×10^2
	Cx. perexiguus	5×10^2
	Cs. longiareolata	1.7×10^3
	Ae. caspius	1.4×10^3

Table 2-6. Cyst production in different hosts

Authors	Species	Production (cysts/individual)
Hembree 1981	*Ae. aegypti*	3.1×10^5
	S. exigua	2.0×10^7
Seif and Rifaat 2001	*Cx. pipiens*	8.1×10^5
	S. littolaris	6.2×10^8
Avery and Undeen 1987b	*H. zea*	7.4×10^8

CHAPTER 3
MATERIALS AND METHODS

Preparation of *Helicosporidium*

Cyst Amplification

The SjHe isolate of *Helicosporidium* from the black fly, *S. jonesi* was collected in 2002 and 2003 by J. Becnel at USDA ARS in Gainesville, Florida (Boucias et al. 2001) SjHe was amplified in *Helicoverpa zea* (Boddie) and extracted on a continuous gradient of Ludox HS40 (Perkin Elmer Life Sciences, Boston MA) following the protocol of Bläske & Boucias (2004). Eggs of *H. zea* were purchased from USDA, ARS, Stoneville, MS. Neonates and larvae were provided with a wheat-germ-based, semi-synthetic diet containing antimicrobial agents and preservatives (Shore and Hale 1965). Neonates were hatched in groups and transferred individually to 24-well plates with diet after reaching the second or third instar. All larvae were maintained at constant conditions (27 ± 1 ˚C, 70 ± 5% RH, photoperiod of 12:12 [L:D] hours). Fourth and fifth instar larvae of *H. zea* were injected with 5 µL of cyst suspension at 2×10^5 cysts/insect. After injection, larvae were transferred to individual diet cups and incubated two weeks as above. Infected pupae and late-instar larvae were homogenized in 200 mL of deionized water with a few crystals of 1-Phenyl 2-Thiourea. The homogenate was filtered twice through paper toweling then subjected to two cycles of low-speed centrifugation (4,000 × *g*, 10 min). The resulting pellet was layered on top of a Ludox gradient formed with a gradient maker from 5% to 60% Ludox in deionized water. After high-speed centrifugation (16,000 × *g*) for one hour, the cyst-containing band was collected and subjected to several cycles of

low-speed centrifugation (4,000 × *g*, 10 min) to remove residual gradient material. All cyst preparations were stored in deionized water at 4°C until use. The number of cysts in each preparation was determined with a hemacytometer using the average of two counts.

In Vitro Dehiscence

Lepidopteran gut extracts were collected from *H. zea* following the protocol of Boucias et al. (2001). Midguts of fourth or fifth instar *H. zea* were dissected out and homogenized gently, then centrifuged at 16,000 × *g* for 15 minutes. The supernatant was removed, passed through a MC centrifugal filter unit (0.45 μm, Millipore Corp., Bedford, MA) and frozen at -20°C in 100 μL aliquots. Rate of dehiscence was evaluated before assays by thawing an aliquot of gut extract and adding 1×10^6 cysts to the gut extract and gently mixing. After incubation at room temperature for 30 minutes, the cysts and filaments were washed in deionized water 2 times using a microcentrifuge at 2,000 × *g* for 10 minutes. The pellet was resuspended in 100 μL of deionized water and the percent dehiscence was quantified with a hemacytometer.

Host Range

Bioassays assessed the activity of SjHe against two mosquito species, *An. quadrimaculatus* and *Ae. aegypti*. Mosquitoes were obtained from colonies maintained at the USDA ARS in Gainesville, FL. *Anopheles quadrimaculatus* were obtained as first instars, while *Ae. aegypti* eggs were obtained from the colony and hatched in 10 mL deionized water with 1 mg of hatch media (finely ground alfalfa and potbelly pig chow mixture (2:1)) under a vacuum. First instar mosquitoes were transferred using a Pasteur pipette and counted into an enamel pan. Larvae were collected on a fine-mesh cloth was and inverted into the bioassay container. For each bioassay,

100 first-instars were placed in a petri dish with 98 mL of deionized water amended with a 1 mL dose of SjHe (treatment) or deionized water (control) and a 1-mL volume of 2% alfalfa and potbelly pig chow mixture (2:1) as a nutritional source (final food concentration 0.2 mg/mL). Larvae were incubated at constant conditions (26 ± 1°C, photoperiod of 12:12 [L:D] hours) for 24 hours then transferred to enamel pans, and water was added to a volume to 500 mL. Food was provided *ad libitum*. After 7 days, the surviving individuals were counted in each pan and a sub-sample of 12 examined randomly for infection under phase-contrast optics. Individuals containing live helicosporidial cells (vegetative or cyst stage) were considered infected. For each species, three replicates were performed at 1×10^5 cysts/mL. Each replicate was accompanied by an untreated control.

Age Susceptibility

All further assays were carried out with *Ae. aegypti*, due to the low control mortality and synchronous development of this species. Large groups of *Ae. aegypti* were hatched and transferred in groups of 500 neonate larvae to enamel pans with 500 mL of water. To obtain second, third, and fourth instars, larvae were reared at constant conditions (26 ± 1°C, photoperiod of 12:12 [L:D] hours) and checked daily for development. Food was provided *ad libitum*. Immediately after molting into the appropriate stage, the larvae were transferred to large petri dishes in groups of 100 as above. The four larval instars were treated at the following dosages. First instars: 1×10^3 to 1×10^5 cysts/mL, second instars: 1×10^4 to 5×10^5 cysts/mL, third instars: 1×10^4 to 1×10^6 cysts/mL, and fourth instars: 1×10^4 to 1×10^6 cysts/mL. An untreated control group was included for each larval instar in each replicate. Larvae were assayed as above

at 7 days post-exposure. If larvae were held long enough for adults to emerge, larvae were transferred to specially constructed mosquito breeders (see Appendix B) rather than enamel pans, allowing for collection of adults. Adults were provided with cotton balls soaked in 10% sucrose solution. When present, surviving pupae and adults were examined for infection at 7 days post-exposure in addition to surviving larvae. The percent infection of surviving pupae and adults was included in the percent infection.

The effect of age of *Ae. aegypti* within the first instar was also examined at 1×10^4 and 5×10^4 cysts/mL. Within two hours of the beginning of hatching, *Ae. aegypti* larvae were counted into groups of 500 as above, placed in 500 mL of deionized water with food, and incubated for 12-24 hours. First instar larvae two hours old were assayed at the same time as 12 hour old and 24 hour old first instar larvae. In each assay, 100 larvae were exposed to Helicosporidia for 24 hours with 0.2 mg/mL of food as above. An untreated control group was included for each larval age and replicate. After 7 days, surviving larvae were counted and a random sample of 12 examined for infection.

Additional experiments were conducted to examine the possibility of latent infections that become detectable only in pupae or adults. Groups of 100 larvae were exposed as first instars to dosages from 5×10^2 to 1×10^4 cysts/mL as above. These were maintained up to 3 weeks post-exposure, fed *ad libitum* at constant conditions ($26 \pm 1°C$, photoperiod of 12:12 [L:D] hours). The number of larvae, pupae, and adults were counted every 2 days. Individuals were assayed for infection at 1, 2, and 3 weeks post-exposure using a random subsample of larvae, pupae, and adults.

Food Concentration Bioassays

The effect of food concentration during exposure to Helicosporidia was examined in first instar *Ae. aegypti*. First instar larvae were counted into groups of 25 and exposed to 1×10^3, 1×10^4 or 1×10^5 cysts/mL with 0.05, 0.1, 0.2 or 0.4 mg/mL of food for 24 hours at constant conditions as above. After 24 hours of exposure to food and Helicosporidia, the larvae were transferred to enamel pans, maintained for 7 days and assayed for infection as above. Mortality was recorded daily.

To further examine the effects of food concentration on ingestion of Helicosporidia, cysts were fixed in 2.5% glutaraldehyde for at least 5 hours, rinsed and labeled with Fluorescein isothiocyanate (FITC) in sodium bicarbonate buffer (pH 9.5) overnight. The resulting fixed, labeled cysts were rinsed 3 times in deionized water in a microcentrifuge at 2,000 × *g* for 10 minutes. Cysts were counted with a hemacytometer and added to 100 mL of deionized water with 25 first-instar *Ae. aegypti* at a dosage of 1×10^5 cysts/mL. Three different food concentrations (0.1, 0.2, 0.4 mg/mL) were tested. After 1, 2, or 4 hours of exposure at 26°C, all 25 larvae were removed and rinsed three times in deionized water. The larvae were homogenized in 0.05% SDS by sonication for 15 seconds, then the labeled cysts in the homogenate were quantified with a hemacytometer. The concentration of cysts in the suspension was used to calculate the average number of cysts ingested per insect.

Host Development

To examine the effect of infection on development time, groups of 100 first instar *Ae. aegypti* larvae were exposed 1×10^4 cysts/mL of SjHe for 24 hours as above. Larvae were rinsed and each transferred into a well of a 24-well plate using a Pasteur pipette. Each well contained 3 mL of water and 0.02 mg/mL of food. Larvae were held at 26°C

and evaluated every day for molting, pupation, or death. Food was provided *ad libitum* until pupation. Two plates of control and two plates of exposed larvae were used. Individuals were assayed for infection after adult emergence.

Statistical Analyses

Where control mortality was not necessary to statistical analyses, mortality of each assay was corrected with the control group mortality using Abbott's formula (Abbott 1921). Statistical analyses were done with the SAS System for Windows (SAS Institute 1999). Percent infection and percent mortality data were subjected to logistic regression using proc genmod. Ingestion of cysts, sex ratios, dehiscence data, and time to pupation were subjected to analysis of variance by the procedure for general linear models (glm) in balanced designs and the procedure for mixed linear models (mixed) in unbalanced designs (Neter et al. 1990; Rao 1998; Younger 1998). The means were separated by the least square means statement (ls means).

CHAPTER 4
RESULTS

Cyst Amplification and Dehiscence

In vitro dehiscence of cysts of SjHe amplified from *H. zea* was highly variable. Dehiscence rates collected from five selected cyst preparations amplified from January to April 2005 varied from 34 to 70% immediately after purification. Percent cyst dehiscence also changed during several weeks of storage at 4˚C (Figure 4-1), rapidly declining 1-2 weeks after purification. After this initial decline, dehiscence rates remained at a low, constant baseline below 10%. In bioassays with *Ae. aegypti*, infection and mortality increased with increasing percent dehiscence (Figure 4-2). Two of the 5 cyst preparations subjected to bioassays had dehiscence rates above 20% (2/9/05 and 3/9/05), and produced an average of 32 ± 15% mortality, and an average percent infection of 53 ± 20%. Other cyst preparations having dehiscence rates below 10% below 10%, and produced an average percent mortality of 17 ± 24% produced average percent infection of 20 ± 11%.

Host Range

Both *Ae. aegypti* and *An. quadrimaculatus* were susceptible to infection with SjHe (Table 4-1). Overall, susceptibility to infection and mortality was higher for *An. quadrimaculatus*. Early mortality, represented by 1-day percent mortality, was higher for *An. quadrimaculatus* in SjHe-treated groups ($df = 1$, $P < 0.0001$, $\chi^2 = 173.2$), but control mortality at 1 day post-exposure was also significantly higher for *An. quadrimaculatus* ($df = 1$, $P = <0.0001$, $\chi^2 = 37.1$). A similar trend held for 7-day mortality (Table 4-2).

At 7 days post-exposure, the hemolymph of infected mosquito larvae was filled with vegetative stages and cysts. Melanized helicosporidial cells were also observed in the head and thorax region of infected mosquitoes. Infection of surviving *An. quadrimaculatus* at 7 days post-exposure was higher than *Ae. aegypti* ($df = 1$, $P < 0.0001$, $\chi^2 = 65.9$). All measurements of mortality and infection were dose-dependent. Melanization, however, was statistically independent of dosage in both species ($df = 1$, $P = 0.6872$, $\chi^2 = 0.16$). Melanization was also independent of infection ($df = 1$, $P = 0.4596$, $\chi^2 = 0.55$). However, percent melanization in surviving individuals 7 days post-exposure was significantly higher in *Ae. aegypti* than *An. quadrimaculatus* ($df = 1$, $P < 0.001$, $\chi^2 = 49.27$).

Age Susceptibility

There was a pronounced decrease in susceptibility with increasing larval age of *Ae. aegypti* (Table 4-3, 4-4). Statistical analysis by probit to obtain LD_{50} values for each age could not be carried out due to lack of consistent mortality or infection above 50% at 7 days post-exposure. However, at 7 days post-exposure to 1×10^5 cysts/mL, first instar larvae had significantly higher mortality and infection rates than second ($df = 1$, $P < 0.0001$, $\chi^2 = 204.88$) or third ($df = 1$, $P < 0.0001$, $\chi^2 = 254.15$) instar larvae. Overall, third and fourth instar larvae were the least susceptible to infection or mortality.

Age within the first instar also had an effect on susceptibility of *Ae. aegypti*. When exposed at 2 hours post-hatch, *Ae. aegypti* exhibited higher early mortality (represented by 3-day percent mortality) and 7-day mortality than 12-hour old larvae or 24-hour old larvae (Table 4-6, 4-7). Susceptibility to infection was also reduced in 24-hour old larvae when compared to 2-hour old larvae ($df = 1$, $P < 0.0001$, $\chi^2 = 17.4$). *Aedes aegypti* larvae exposed to 5×10^3 cysts/mL 24 hours after hatching were resistant to infection.

The percent infection of surviving *Ae. aegypti* larvae at 7 days post-exposure did not differ significantly from the percent infection in larvae, pupae, or adults sampled at 2 or 3 weeks post-exposure (Table 4-8, 4-9). Infection rates were low for live pupae and adults sampled at 3 weeks post-exposure. Of 141 total adults examined, 7 were found infected. Of the 25 total pupae examined, only one was found infected. Mortality was high in the first 3 days post-exposure, and again increased after the second week post-exposure (Figure 4-3). Dead, infected larvae and pupae were recovered 7-14 days post-exposure. The onset of pupation and adult emergence occurred at the same time for control and SjHe-treated groups (Figure 4-4 and 4-5). However, groups treated with SjHe had a higher proportion of adults at 3 weeks post-exposure when compared with untreated controls, due to the death of larvae and pupae in SjHe-treated groups. There were more than twice as many adult males as females in control groups at 3 weeks post-exposure. Sex ratios in groups treated with SjHe were slightly weighted toward females, but not significantly ($df = 4$, $P = 0.4711$, $F = 0.99$) (Table 4-10).

Food Concentration Bioassays

Increased food availability during exposure to SjHe decreased mortality and infection in *Ae. aegypti* larvae. Total mortality increased 1-3 days post-exposure, leveling off after 3 days post-exposure such that, in most food and dose combinations, 3-day mortality accounted for 50-90% of total mortality at 7 days post-exposure (Table 4-11). At two days post-exposure, mortality was significantly affected by both dosage and food level ($df = 6$, $P = 0.0025$, $F = 4.63$) (Figure 4-6). The relationship between food and mortality at 7 days post-exposure was significant at the 1×10^3 dosage ($df = 3$, $P = 0.0145$, $F = 3.98$) (Table 4-12). The effect of food level on infection at seven days was only statistically analyzed for the 1×10^3 dosage due to high mortality (~100%) in

the other treatments. At 1×10^3 cysts/mL dosage, increased food levels decreased infection ($df = 3$, $P = 0.0278$, $F = 5.19$) (Table 4-13).

The number of fixed, FITC-labeled cysts ingested by first instar *Ae. aegypti* decreased with increasing food availability ($df = 2$, $P = 0.0061$, $F = 6.31$) (Table 4-14). The number of cysts ingested per insect was not significantly different for 1, 2, or 4 hours post-exposure ($df = 2$, $P = 0.5719$, $F = 0.57$). However, ingestion rates were highly variable.

Host Development

Infection with SjHe had no effect on time to pupation for individually-reared *Ae. aegypti*. Of the 48 exposed larvae transferred into individual wells, 33 survived to pupation. The infection rate in these pupae was 63%. Time to pupation was not significantly delayed in infected individuals when compared with uninfected individuals ($df = 4$, $P = 0.9010$, $F = 0.26$). Average time to pupation in infected larvae was 9.4 ± 1.7 days, while the average time to pupation for control larvae was 8.6 ± 1.5 days.

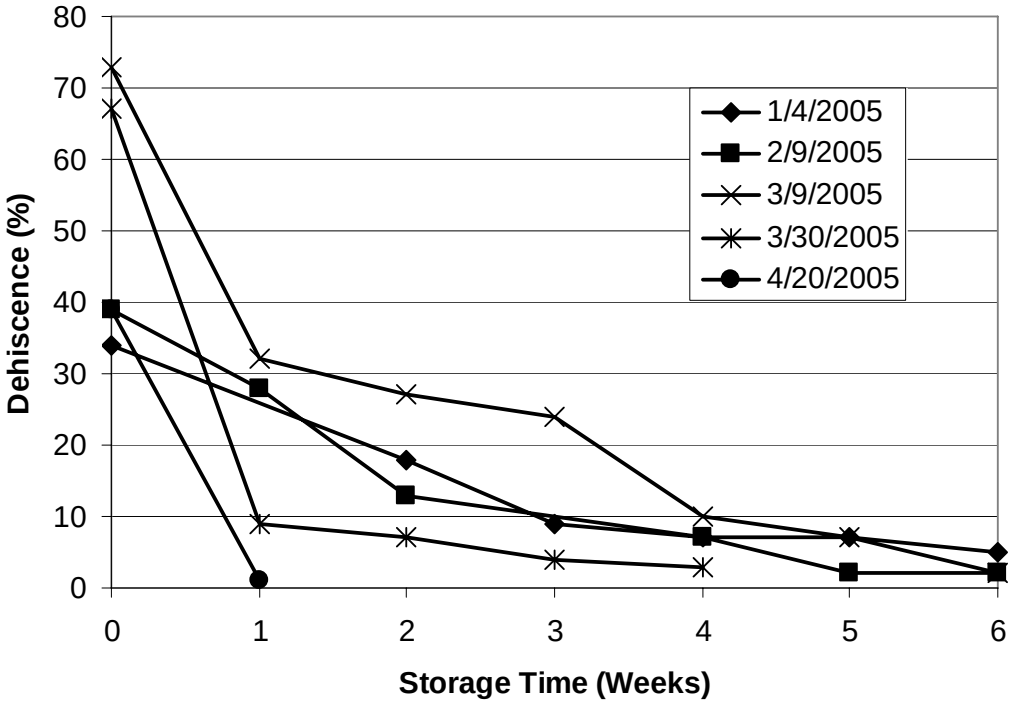

Figure 4-1. Percent dehiscence of five cyst preparations purified January through April 2005. After purification, cyst preparations were stored at 4˚C. Cyst preparations were assayed for dehiscence immediately after purification (0 weeks) and each week after purification. Cysts were treated with lepidopteran gut extracts, incubated at room temperature for 30 minutes, then the percent of filamentous cells released was counted with a hemacytometer to obtain percent dehiscence. Data provided by V. Lietze.

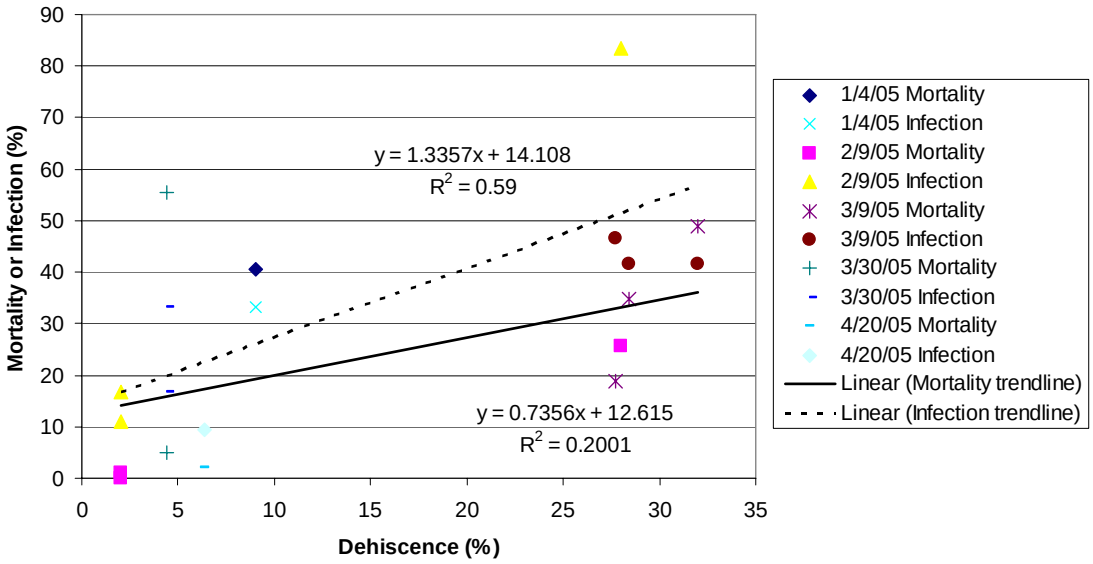

Figure 4-2. Regression of percent corrected mortality and infection on percent dehiscence of cyst preparation at time of exposure. Each point represents a single bioassay with one of the five isolates purified from Jaunuary to April 2005. Groups of 100 first instar *Ae. aegypti* were exposed for 24 hours to 1×10^4 cysts/mL. Percent mortality was obtained at 7 days post exposure, using Abbott's correction for control mortality. Percent infection was obtained by examining a subsample of surviving larvae at 7 days post-exposure.

Table 4-1. Average ± SD results of assays with *An. quadrimaculatus* and *Ae. aegypti* 7 days after exposure to 1×10^4 cysts/mL. Infection and melanization were measured in a subsample of surviving larvae 7 days after exposure.

Species	Dose (cysts/mL)	Day 1 Percent Mortality	Day 7 Percent Mortality	Percent Infection	Percent Melanization
An. quadrimaculatus	Control	15 ± 30 (5) [a]	38 ± 28 (7)	0 ± 0 (3, 36) [b]	0 ± 0 (3, 36)
	1×10^3	34 ± 25 (4)	78 ± 10 (4)	69 ± 43 (4, 43)	18 ± 27 (4, 43)
	1×10^4	34 ± 33 (6)	72 ± 13 (5)	84 ± 20 (10, 111)	8 ± 14 (10, 111)
Ae. aegypti	Control	1 ± 2 (5)	5 ± 4 (6)	0 ± 0 (3, 36)	0 ± 0 (3, 36)
	1×10^3	1 ± 1 (3)	11 ± 3 (3)	11 ± 13 (3, 36)	44 ± 13 (3, 36)
	1×10^4	10 ± 6 (6)	57 ± 19 (8)	33 ± 30 (6, 72)	57 ± 14 (6, 72)

[a] Number of replicates, each consisting of 100 larvae.
[b] Total number of individuals examined across all replicates appears in parentheses after number of replicates.

Table 4-2. Logistic regression of host range bioassay data (proc genmod).

Response	Variable	*df*	X^2	*P*
Day 1 Mortality	Dose (1×10^3 vs 1×10^4)	1	6.1	0.0136
	Species (Control)	1	37.1	<0.0001
	Species (SjHe)	1	173.2	<0.0001
Day 7 Mortality	Dose (1×10^3 vs 1×10^4)	1	60.5	<0.0001
	Species (Control)	1	148.5	<0.0001
	Species (SjHe)	1	214.7	<0.0001
Percent Infection	Dose	1	14.3	0.0002
	Species	1	65.9	<0.0001
Percent Melanization	Dose	1	0.16	0.6872
	Species	1	49.27	<0.0001
	Infection	1	0.55	0.4596

Table 4-3. Average ± SD corrected percent mortality at 7 days post-exposure for four instars of *Ae. aegypti* at five dosages of *Helicosporidium*.

	7-Day Mortality Dosage (cysts/mL)			
Instar	1×10^3	1×10^4	1×10^5	1×10^6
1	7 (1) [a]	23 ± 23 (4)	83 ± 24 (3)	*nt*
2	1 (1)	5 ± 4 (3)	19 ± 22 (3)	*nt*
3	2 (1)	3 (1)	4 ± 5 (4)	12 (1)
4	*nt* [b]	*nt*	0 (1)	0 (3)

[a] Number of replicates appear in parentheses. Each replicate consisted of 100 larvae. Abbott's correction for control mortality was used.
[b] Not tested

Table 4-4. Average ± SD corrected percent infection of surviving larvae at 7 days post-exposure for different instars of *Ae. aegypti* at five dosages of Helicosporidia.

	Percent Infection Dose (cysts/mL)			
Instar	1×10^3	1×10^4	1×10^5	1×10^6
1	25 (1, 12) [a]	42 ± 30 (4, 48)	75 ± 25 (3, 17)	*nt*
2	0 (1, 12)	6 ± 10 (3, 36)	6 ± 5 (3, 36)	*nt*
3	0 (1,12)	0 (1, 12)	2 ± 4 (4, 48)	25 (1, 12)
4	*nt* [b]	*nt*	8 (1, 12)	14 ± 17 (3, 36)

[a] The number of replicates appears in parentheses, followed by the total number of larvae examined across all replicates. Each replicate consisted of 100 larvae.
[b] Not tested

Table 4-5. Logistic regression of age susceptibility by instar (proc genmod).

Response	Variable	*df*	χ^2	*P*
Day 7 Mortality	Instar (1 vs 2)	1	204.88	<0.0001
	Instar (1 vs 3)	1	254.15	<0.0001
	Instar (2 vs 3)	1	36.41	<0.0001
Percent Infection	Instar (1 vs 2)	1	9.45	0.0021
	Instar (1 vs 3)	1	21.18	<0.0001
	Instar (2 vs 3)	1	9.60	0.0019

Table 4-6. Mean ± SD percent mortality and infection in 2, 12, and 24-hour old *Ae. aegypti* after exposure to SjHe and at 5×10^3 and 1×10^4 cysts/mL.

Dose (cysts/mL)	Age (hours post-hatch)	N[a]	Day 3 Percent mortality[c]	Day 7 Percent mortality[c]	Percent Infection[b]
5×10^3	2	3	35 ± 26	49 ± 34	40 ± 13 (36)
	12	4	9 ± 14	22 ± 37	10 ± 21 (48)
	24	3	2 ± 3	2 ± 3	0 ± 0 (36)
1×10^4	2	3	63 ± 9	80 ± 15	25 ± 22 (26)
	12	4	29 ± 33	39 ± 43	29 ± 34 (39)
	24	3	28 ± 43	27 ± 45	19 ± 34 (36)

[a] Number of replicates, consisting of 100 larvae each.
[b] Total number of larvae examined across all replicates appears in parentheses.
[c] Abbott's correction for control mortality was used.

Table 4-7. Logistic regression of *Ae. aegypti* bioassay data for age and dose.

Response	Variable	*df*	χ^2	*P*
Day 3 Mortality	Dose	1	87.7	<0.0001
	Age (2h vs 12h)	1	29.2	<0.0001
	Age (2h vs 24h)	1	140.0	<0.0001
	Age (12h vs 24h)	1	39.1	<0.0001
Day 7 Mortality	Dose	1	61.1	<0.0001
	Age (2h vs 12h)	1	17.6	<0.0001
	Age (2h vs 24h)	1	247.6	<0.0001
	Age (12h vs 24h)	1	131.8	<0.0001
Percent Infection	Dose	1	2.24	0.1346
	Age (2h vs 12h)	1	2.8	0.0930
	Age (2h vs 24h)	1	17.4	<0.0001
	Age (12h vs 24h)	1	6.06	0.0138

Table 4-8. Mean ± SD infection at 1, 2, and 3 weeks post-exposure of *Ae. aegypti* exposed as first instars to four dosages of SjHe.

		Mean Percent Infection		
Dose (cysts/mL)	Week Post-Exposure	Larvae	Pupae	Adults
5×10^2	1	4 ± 6 (24)[a]	*np*[b]	*np*
	2	0 (12)	*np*	0 (12)
	3	11 ± 16 (21)	17 ± 24 (6)	0 (12)
1×10^3	1	14 ± 13 (36)	*np*	*np*
	2	0 (9)	0 (3)	0 (12)
	3	12 ± 7 (37)	0 (11)	0 ± 0 (33)
5×10^3	1	46 ± 41 (24)	*np*	*np*
	2	27 (12)	0 (1)	0 (12)
	3	58 ± 59 (13)	0 ± 0 (4)	4 ± 6 (24)
1×10^4	1	58 ± 30 (36)	*np*	*np*
	2	33 (9)	0 (3)	0 (9)
	3	*np*	*np*	33 ± 42 (27)

[a] Three replicates were conduced at 1 and 3 weeks, one replicate at 2 weeks, total number of individuals examined across all replicates appears in parentheses.
[b] Not present.

Table 4-9. Logistic regression of percent infection at 1, 2, and 3 weeks post-exposure.

Variable	*df*	χ^2	*P*
Week (1 vs 2)	1	0.00	0.9998
Week (1 vs 3)	1	1.31	0.2519
Week (2 vs 3)	1	0.00	0.9998

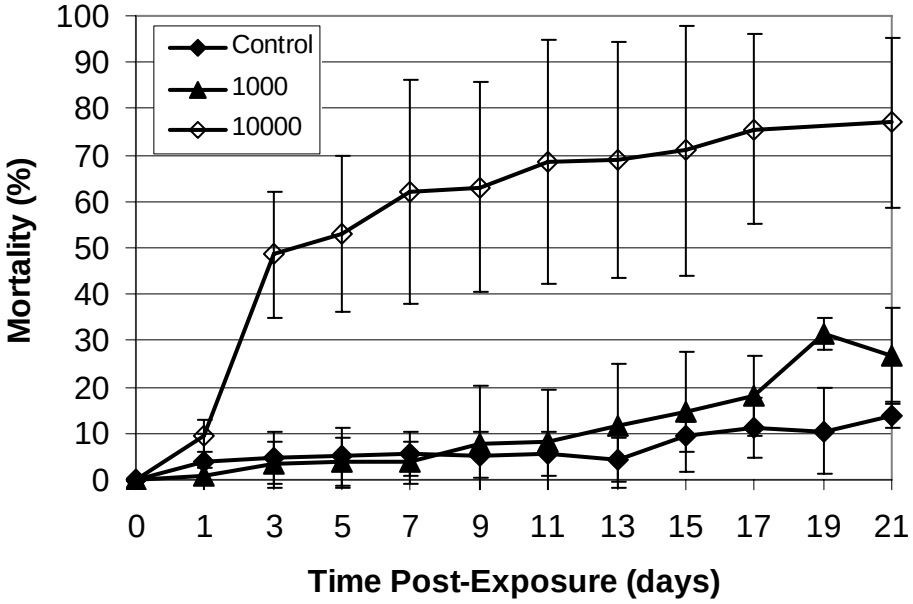

Figure 4-3. Percent mortality over time of first instar *Ae. aegypti* exposed to two dosages of SjHe. Two replicates were conducted.

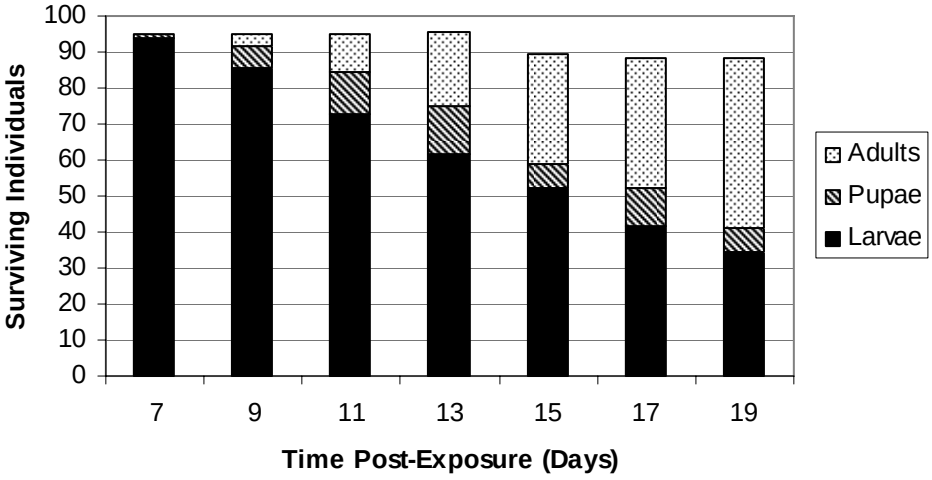

Figure 4-4. Average control proportion of larvae, pupae, and adults of surviving *Ae. aegypti* 1-3 weeks post-exposure.

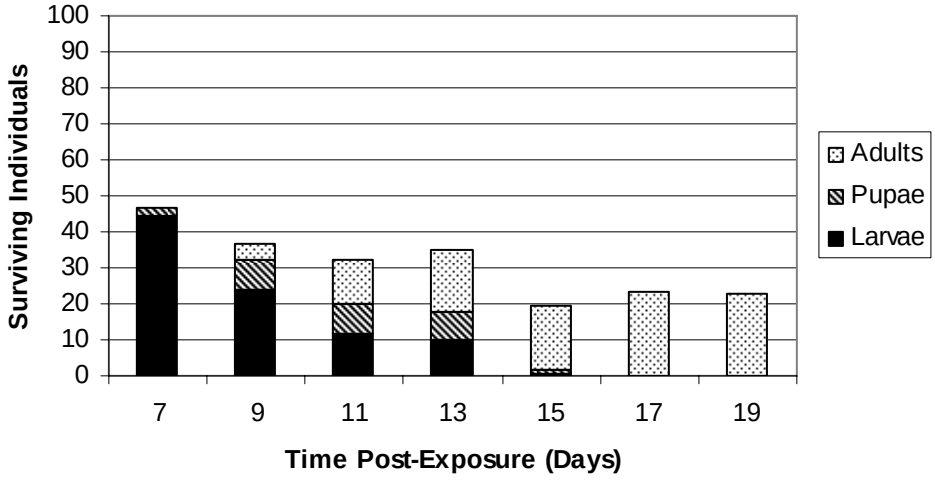

Figure 4-5. Average proportion of larvae, pupae, and adults of surviving *Ae. aegypti* at 1-3 weeks post exposure to 1×10^4 cysts/mL of SjHe.

Table 4-10. Mean ± SD adult male:female ratio of *Ae. aegypti* at 3 weeks post-exposure to 2 dosages of SjHe.

Dosage (cysts/mL)	Mean M:F Ratio[a]
0	2.1 ± 0.9a
1×10^3	2.3 ± 1.5a
1×10^4	0.8 ± 0.2a

[a] Means followed by different letters were significantly different (proc glm, lsmeans; $P < 0.05$)

Table 4-11. Corrected percent mortality over time of *Ae. aegypti* at three dosages of SjHe and four food levels

		Percent Mortality Days post exposure						
Dosage (cysts/mL)	Food	1	2	3	4	5	6	7
1×10^3	0.05	4 ± 0.2 [a]	22 ± 6	35 ± 25	43 ± 18	51 ± 27	53 ± 30	54 ± 36
	0.1	3 ± 3	12 ± 17	14 ± 20	14 ± 22	22 ± 31	26 ± 37	30 ± 30
	0.2	3 ± 2	3 ± 2	3 ± 5	6 ± 5	11 ± 10	9 ± 9	21 ± 22
	0.4	2 ± 3	2 ± 3	6 ± 9	6 ± 9	6 ± 9	6 ± 9	6 ± 9
1×10^4	0.05	12 ± 5	96 ± 6	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	0.1	22 ± 6	81 ± 10	87 ± 5	92 ± 0	93 ± 2	96 ± 4	98 ± 2
	0.2	7 ± 6	53 ± 9	67 ± 11	84 ± 3	84 ± 3	92 ± 7	95 ± 6
	0.4	10 ± 14	23 ± 3	40 ± 9	54 ± 24	57 ± 32	62 ± 30	72 ± 23
1×10^5	0.05	37 ± 27	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	0.1	35 ± 21	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	0.2	20 ± 1	96 ± 6	98 ± 3	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	0.4	16 ± 6	92 ± 0	94 ± 3	94 ± 3	95 ± 5	95 ± 6	97 ± 5

[a] Three replicates were performed, Abbott's correction for control mortality was used

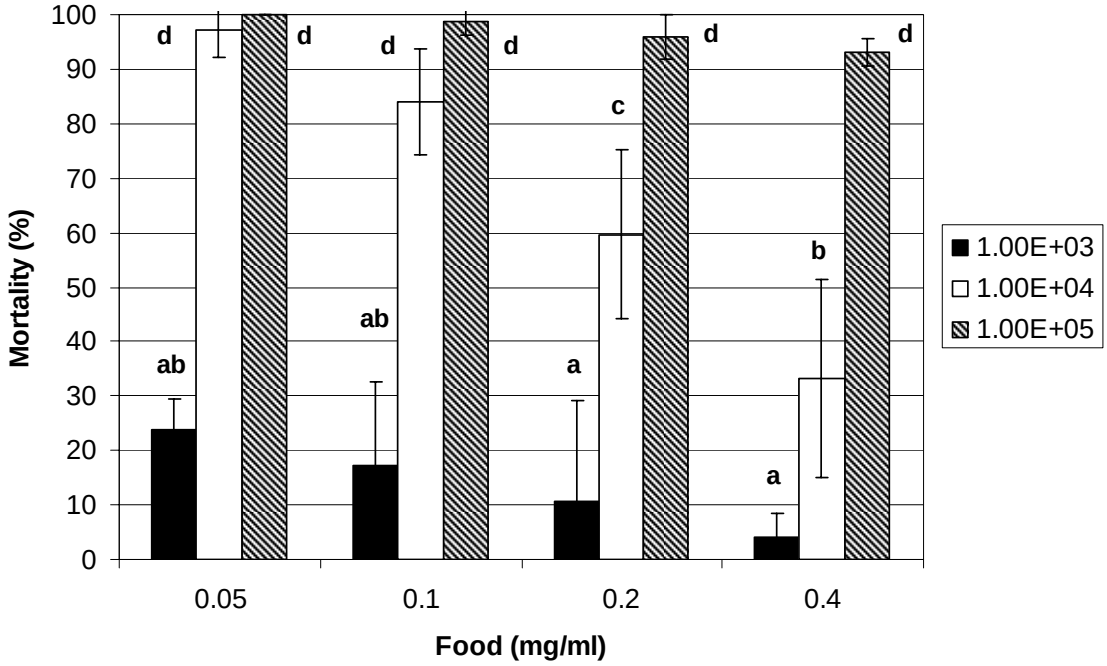

Figure 4-6. Percent mortality at 2 days post-exposure of first instar *Ae. aegypti* exposed to three dosages of Helicosporidia and four food levels. Three replicates were performed. Means followed by different letters were statistically different (proc glm, lsmeans; $P < 0.05$). Abbott's correction for control mortality was used.

Table 4-12. Mean (± SD) corrected percent mortality in *Aedes aegypti* 7 days after exposure to SjHe at four different food levels and three dosages of helicosporidia.

	7-day Percent Mortality Food (mg/mL)			
Dosage (cysts/mL)	0.05	0.1	0.2	0.4
1×10^3	43 ± 36a [a b]	27 ± 25ab	22 ± 19ab	3 ± 6b
1×10^4	93 ± 14a	91 ± 16a	77 ± 39a	54 ± 40a
1×10^5	100 ± 0a	100 ± 0a	99 ± 2a	86 ± 23a

[a] Three replicates were performed, consisting of 25 larvae each, Abbott's correction for control mortality was used.
[b] Means followed by different letters are statistically different within each dose (proc glm, lsmeans; $P < 0.05$).

Table 4-13. Mean (± SD) infection rates in surviving *Ae. aegypti* 7 days after exposure to three dosages of SjHe at four different food levels. Three replicates were performed. The total number of individuals examined across all replicates appears in parentheses. Means followed by different letters were statistically different (proc glm, lsmeans; $P < 0.05$).

	Percent Infection Food (mg/mL)			
Dosage (cysts/mL)	0.05	0.1	0.2	0.4
1×10^3	72 ± 25a (24) [a b]	52 ± 25ab (34)	34 ± 8bc (41)	14 ± 10c (36)
1×10^4		100 ± 0 (2)	83 ± 24 (5)	67 ± 37 (18)
1×10^5			100 (1)	0 (2)

[a] Three replicates were performed, consisting of 25 larvae each, Abbott's correction for control mortality was used.
[b] Means followed by different letters are statistically different within each dose (proc glm, lsmeans; $P < 0.05$).

Table 4-14. Mean (± SD) fixed, FITC-labeled cysts ingested per *Ae. aegypti* larva exposed to 1×10^5 cysts/mL at three food levels. Means followed by different letters were statistically different (proc glm, lsmeans; $P < 0.05$).

Food (mg/mL)	Cysts per insect
0.1	$4.2 \pm 4.4 \times 10^3$a
0.2	$3.1 \pm 3.0 \times 10^3$ab
0.4	$2.0 \pm 2.0 \times 10^3$b

CHAPTER 5
DISCUSSION

Highly variable bioassay data seem to be the norm with many *Helicosporidium* isolates. Fukuda et al. (1976) found that infection rates of *An. quadrimaculatus* exposed to a mosquito isolate varied between 17 and 93% among four replicates. Bläske and Boucias (2004) found that oral challenge of three noctuid species with an isolate of *Helicosporidium* from an aquatic weevil produced either no infection or only 50% infection. However, injection of the noctuids with *Helicosporidium* consistently resulted in 100% infection. Initial infection events thus may be the source of the variability in bioassay data. In our study, cyst viability has also been implicated to play a role in the variable bioassay results produced with different cyst preparations.

Previous studies examining cyst viability have used a standardized mosquito bioassay. Hembree (1981), for example, exposed groups of 100 first instar 24-hour old *Ae. aegypti* to a range of dosages of *Helicosporidium* after storage or treatment with various environmental factors, measuring infection rate as the response variable. Avery and Undeen (1987b), on the other hand, used groups of 150 first instar *An. quadrimaculatus* exposed to a range of dosages of *Helicosporidium* for 24 hours, measuring 72-hour mortality as the response variable. In vitro methods for assessing cyst viability have not been tested before. Since dehiscence is necessary to initiate infection and directly related to viability, dehiscence rate is a logical choice for assessing cyst viability. Variation in dehiscence rate of SjHe between cyst preparations and decline of dehiscence rates during storage may account for the high variability in the bioassay data.

However, the relationship between dehiscence and mortality and infection was not very strong, indicating that in vitro dehiscence may not be the best indicator of cyst viability. Also, there may be factors other than cyst viability influencing variation in the bioassay data.

Helicosporidium has a wide host range. Natural infections have occurred in mites, cladocerans, Coleoptera, Collembola, Diptera, Lepidoptera, Oligochaeta, and parasitic trematodes. *Helicosporidium* is not highly host-specific. For example, Kellen and Lindegren (1973) transmitted a *Helicosporidium* isolate obtained from *Carpophilus mutilatus* Erichson to 9 other species of Coleoptera, 6 species of Lepidoptera, 3 species of mites, and 1 species of Diptera. There are only five mosquito host records for *Helicosporidium*, but *Helicosporidium* from mosquitoes has been transmitted to 25 other species of mosquitoes, three species of Coleoptera, and three species of Lepidoptera.

Previous SjHe host range results indicated that *Ae. aegypti* was a less suitable host than *An. quadrimaculatus* (Conklin et al. 2005). For example, in previous studies, SjHe infection rates were below 20% for first instar *Ae. aegypti* treated with 1×10^5 cysts/mL. In our study, the infection rate of first instar *Ae. aegypti* exposed to 1×10^5 cysts/mL was 75 ± 25%. Also, in previous studies, mortality of first instar *Ae. aegypti* exposed to 1×10^5 cysts/mL SjHe was less than 10%. In our study, mortality of first instar *Ae. aegypti* exposed to 1×10^5 cysts/mL was 83 ± 24%. In addition, Conklin et al. (2005) reported that the SjHe isolate did not cause melanization in mosquito hosts, whereas the isolate from an aquatic weevil (CsHe) did cause melanization. In our study, melanization was more prevalent in *Ae. aegypti* than *An. quadrimaculatus*, possibly indicating that *Ae. aegypti* are still less suitable hosts for *Helicosporidium* than *An. quadrimaculatus*. It is

difficult to say what might have caused these differences. Isolated from a blackfly in 2003, SjHe has been continuously amplified in lepidopteran hosts. The Helicosporidial cells used by Conklin et al. (2005) had been amplified in lepidopterans for less than a year, but the Helicosporidial cells used in our study had been in culture in *H. zea* for 1-2 years. Continued selection by amplification in a heterologous host may have caused a phenotypic change in the isolate over time. Avery and Undeen (1987b) described changes in cyst diameter of a pond-water isolate after one passage through a lepidopteran host. Hembree (1981), likewise, amplified a mosquito isolate in *S. exigua* and reported a loss of infectivity after one passage through this heterologous host. Rather than exhibiting a decrease in infectivity, the SjHe isolate appears to have increased infectivity toward *Ae. aegypti*. There is also a possibility that the SjHe isolate was contaminated with the weevil isolate (CsHe) used by Conklin et al. (2005).

Age-dependent resistance to pathogens is common in insects as well as many other organisms. There are several possible reasons for decreased susceptibility due to age. Age may alter ingestion or feeding as the larvae become satiated and cease feeding or begin molting into the next instar. In this study, 24-hour-old first instar larvae molted into the second instar during the exposure period, possibly resulting in reduced ingestion. Older larvae also have had time to establish a compliment of immune peptides and phagocytic hemocytes that may kill invasive microorganisms. In mosquito larvae, the peritrophic matrix may also be a key to pathogen resistance. Mosquito larvae have a highly structured peritrophic matrix secreted by a ring of cells in the cardia and extending through the entire midgut. The peritrophic matrix is already present in neonate *Ae. aegypti*, but it is thinner, without the strengthening fibers present in the peritrophic

matrix of later instars. Thickening of the peritrophic matrix occurs as the larvae age and feed (Richards and Richards 1971).

Decreasing susceptibility with age has been reported for three other *Helicosporidium* isolates. Fukuda et al. (1976) reported a 3-fold reduction in infection rate from larvae 1 to 3 days old. Hembree (1981) found that both instar and age during the first instar had an effect on infectivity and mortality. In Hembree's (1981) study, there was a 3-fold difference in IC_{50} between first and second instars of *Ae. aegypti*. Seif and Rifaat (2001) reported that the IC_{50} of second instar *Cx. pipiens* was only 1.2 times more than the first instars. In our study, there was an 8 to 13-fold difference in infection rate between the first and second instars of *Ae. aegypti*. The results for the effect of age within the first instar in this study were similar to those of Hembree (1981), who found a 2-fold difference in infection rate of 2 and 24-hour old *Ae. aegypti* at 5×10^2 cysts/mL. However, the decrease in susceptibility between 2 and 24-hour old *Ae. aegypti* in our study was more pronounced, decreasing from 40 to 0% from 2 to 24-hours of age at a dosage of 5×10^3 cysts/mL. Increasing the dosage of SjHe minimized the effect of first instar age on susceptibility, indicating that the decrease in susceptibility due to age was not complete.

Infection with *Helicosporidium* reaches its peak just after 7 days post-exposure. Fukuda et al. (1976) reported that infection could be detected at 4 days post-exposure and that the maximum number of infected mosquitoes was collected 9-10 days post-exposure. Hembree (1981) found that virtually all infections could be detected 4 days after infection, and that 10-14 day-old larvae were optimum for cyst purification. Seif and Rifaat (2001) reported 5 days post-exposure for the first detectable infections, and that

the maximum number of infected larvae was collected 8-10 days post-exposure. All of the above authors noted that mortality occurred in the late fourth instar, pupae, and adults. The pathological effects of *Helicosporidium* infection manifest themselves at the larval-pupal interface in lepidopteran hosts also. Bläske and Boucias (2004) found that infected noctuids successfully pupated, but most died as pupae. A small number of infected larvae emerged as infected adults. Infected noctuiid adults and pupae were often malformed. In bioassays with SjHe, percent infection did not change significantly after 1 week post-exposure, and most infected insects died late in the fourth instar, at the larval-pupal interface. No malformation of pupae or adults was observed in SjHe-treated *Ae. aegypti.* Sex-ratio in infected noctuiids was not different from controls (Bläske and Boucias 2004). Sex ratio of exposed *Ae. aegypti* also did not differ from controls. The minor female-bias in the sex ratio of *Ae. aegypti* exposed to SjHe is due to the high mortality in these groups.

Infected larvae that successfully pupated and emerged as adults were rare in this study. Horizontal transmission appears to be the most likely form of transmission for *Helicosporidium*. Bläske and Boucias (2004) found that *Helicosporidium* infection could be vertically transmitted by infected noctuiid adults. However, the infection rate in the offspring was very low, and the ovarian tissue itself was not found to be infected. The role of vertical transmission in mosquitoes is unknown. The only study of vertical transmission in mosquitoes was by Fukuda et al. (1976), which examined 2060 larvae from 17 egg rafts of infected *Cx. salinarius*, but found no infection. Natural horizontal transmission of *Helicosporidium* from infected individuals to uninfected individuals of the same species has not yet been documented with any host species. However, Hembree

(1981) infected *Toxorhynchites splendens* (Wied.) by feeding the predatory larvae *Helicosporidium*-infected *Cx. pipiens*. Once cysts are produced in the host, they must be released into the environment in order to be ingested by the next host, but no mechanism of cyst release has been found (Bläske-Lietze et al. in press).

While death of infected larvae during the fourth instar appears to be common for *Helicosporidium* infections, early mortality accounted for most of the total mortality at 7 days post exposure. High early mortality due to exposure to SjHe in this study and also observed by Conklin et al. (2005) has only been reported for one other isolate of *Helicosporidium*. Avery and Undeen (1987b) found that a pond water isolate of *Helicosporidium* caused high mortality in first instar larvae at 72-hours post exposure. The pond water isolate also caused melanization in mosquitoes. Since early mortality causes the death of the host before cyst production, terminating the infection cycle, the authors suggested that mosquitoes were not natural hosts of the pond water isolate.

Increasing the food during exposure to SjHe decreased the amount of cysts ingested, as indicated with fixed, FITC-labeled cysts. However, decreased ingestion may not completely explain decreased infection. In addition to reduced ingestion, plant chemicals present in the mosquito diet may have damaged the ingested helicosporidial cells. Alfalfa, a component of the larval food, produces isolfavonoid phytoalexins, defensive compounds that exhibit antimicrobial activity in vitro (He and Dixon 2000). Differences in food composition may account for variation in bioassay results from previous studies. For example, Hembree (1981) used a high-protein mixture of yeast, liver powder, and hog chow as larval food, while Fukuda et al. (1976) used rabbit chow, and Seif and Rifaat (2001) used powdered tropical fish food.

In our study, there was no effect of infection on development. However, there are other reports that *Helicosporidium* can influence development in other hosts. For example, Conklin et al. (2005) found that the mean head capsule measurements of *Ae. aegypti* exposed to SjHe and reared in groups were smaller than the control larvae. However, this approach failed to take into account the effect of group size on host development. Individual rearing eliminates this larval density factor, providing each larva with nearly ideal conditions for growth. It is possible that, in groups, larval density and competition between larvae for food increases the cost of infection, thus increasing the effect of infection on development. In support of this, Bedhomme et al. (1999) found that *Ae. aegypti* infected with a microsporidian developed more slowly when placed in groups than individually-reared larvae.

Utilization of the time to pupation as a measurement of development may not have been adequate. Bläske and Boucias (2004) reported that the effect of infection on noctuid development varied by species and measurement of development. Pupal weight, time to pupation, and time to eclosion each provided a different profile of the effect of *Helicosporidium* infection on development. Also, analysis of time to pupation in SjHe-infected *Ae. aegypti* would have been improved by recording pupation more than once every 24 hours. With such a short larval period, the difference in development between infected and control mosquitoes may have been less than 1 day. However, Hembree (1981) reported that pupation was delayed 1-2 days in infected *Ae. aegypti* and pupation was lengthened. Different rearing conditions may have caused slower development in his infected larvae. The length of time to development (pupation or adulthood) can also have an effect on a pathogen's ability to influence host development. The length of the larval

period of *Ae. aegypti* at 26 ˚C is 7-10 days, while the length of the larval period of the weevil *D. abbreviatus* is 100 days or more (Conklin et al. 2005), providing the pathogen a greater opportunity to influence the development of the host. Accordingly, there was a pronounced effect on host development in *D. abbreviatus* treated with SjHe.

While *Ae. aegypti* and *An. quadrimaculatus* support vegetative growth of SjHe, several aspects of the interaction between SjHe and mosquitoes indicate that host suitability is low for these species. The IC_{50} for first-instar *An. quadrimaculatus* was between 1×10^4 and 1×10^5 cysts/mL (Conklin et al. 2005), and for *Ae. aegypti* the IC_{50} of first-instars was also in the range of 1×10^4 cysts/mL. The dose-response of two culicid isolates have been examined using similar methods, with much lower IC_{50}'s than this study. Hembree (1981) found that the IC_{50} of first instar *Ae. aegypti* exposed to a mosquito isolate from Thailand was approximately 1×10^3 cysts/mL. Seif and Rifaat (2001) found that for first instar *Cx. pipiens* exposed to another mosquito isolate, the IC_{50} was 1.9×10^2 cysts/mL. High initial mortality of *Ae. aegypti* and *An. quadrimaculatus* exposed to SjHe limited the number of infected, cyst-producing larvae recovered later. Cyst production after death also does not appear to occur in other hosts of *Helicosporidium*. Bläske-Lietze et al. (in press) have found that in *H. zea*, vegetative reproduction halts after the host's death, and further cyst differentiation does not occur in the dead host. This interaction between a pathogen and the non-host species did not benefit either the host or the pathogen. The host had an acute reaction, causing death and shutting down pathogen reproduction, while the pathogen, having prematurely killed its host, was unable to complete its life cycle.

The host range of a single *Helicosporidium* isolate may be deceptively broad, where many species can be infected, but only a few support a high level of replication and transmission, representing true host species. The sensitivity of the bioassay system of SjHe and mosquitoes to cyst viability, minor changes in host age, and food availability also indicate a poorly adapted host-pathogen interaction.

APPENDIX A
MEASUREMENT DATA

Table A-1. Compiled cyst measurements.

References	Size (µm)	Stain	Fixation	Host
Keilin 1921	5 - 6	None	None	*Dasyhelea obscura*
Weiser 1970	3 - 4.5	Iron-haematoxylin	Carnoy's fluid, paraffin	*Hepialis pallens*
Weiser 1970	4.5 - 5	Iron-haematoxylin	Schaudinn's fluid + 1% HAc	*Dasyhelea obscura*
Hembree 1979	5.5	Giemsa	Methanol	*Cx. quinquefasciatus*; *Ae. aegypti*
Hembree 1979	5.9	None	None	*Cx. quinquefasciatus*; *Ae. aegypti*
Kellen & Lindegren 1974	5.03 ± 0.28	Giemsa	Methanol	*Paramyelois transitella*
Kellen & Lindegren 1974	5.59 ± 0.26	None	None	*Paramyelois transitella*
Sayre & Clark 1978	5.6 ± 0.052	Giemsa	None	*Daphnia magna*
Pekkarinen 1993	5.31 ± 0.04	None	Glutaraldehyde	Bucephalid trematode
Pekkarinen 1993	4.99 ± 0.04	Stained	None	Bucephalid trematode
Pekkarinen 1993	4.91 ± 0.05	Stained	None	Bucephalid trematode
Pekkarinen 1993	4.92 ± 0.06	Stained	None	Bucephalid trematode
Pekkarinen 1993	3.71 - 5.14	TEM	TEM	Bucephalid trematode
Boucias et al. 2001	6.5 ± 0.2 x 5.9 ± 0.3	None	None	*Simulium jonesi*
Boucias et al. 2001	6.2 ± 0.3 x 5.9 ± 0.1	None	None	*Helicoverpa zea*
Avery & Undeen 1987a	7.19 ± 0.15	Giemsa	None	Collembolan
Avery & Undeen 1987a	5.58 ± 0.03	None	None	*Helicoverpa zea*
Avery & Undeen 1987b	8.91 ± 0.12	None	None	Collembolan

Table A-2. Compiled filamentous cell measurements

Authors	Size (μm)	Stain	Mount	Host
Keilin 1921	60 - 65 x 1	None	None	*Dasyhelea obscura*
Kellen & Lindegren 1974	50.4 ± 3.32	None	Saline	*Paramyelois transitella*
Sayre & Clark 1978	61.7 ± 2.44	Giemsa	None	*Daphnia magna*
Boucias et al. 2001	37 ± 4.3 x 0.9 ± 0.13	None	None	*Simulium jonesi*

APPENDIX B
CONSTRUCTION OF MOSQUITO BREEDERS

When rearing mosquitoes to adulthood, it becomes necessary to find a way to have both water for the larvae and pupae and space for the adults to be collected after emergence. Placing the larval rearing pan into a large screen cage is one simple way to address this problem. However, screen cages are bulky and difficult to sterilize, and collecting adults from such a large space can be a challenge. Many "mosquito breeder" products that address this problem are available through mail order, but these usually cost $10-15 each. A mosquito breeder has a relatively simple design, however, and can be constructed from materials that most laboratories have on hand. In this series of experiments, mosquito breeders were constructed from 16 oz clear, plastic food containers with tight-fitting lids ("Del-Pak" by Reynolds Grant Park, IL). These containers are light, durable, dishwasher-safe, stackable, water-tight, easy to cut with a razorblade, and recyclable. The following is a protocol for constructing mosquito breeders.

Materials

- Three 16 oz plastic tubs
- Two lids for tubs
- One 5x5 in piece of green tulle
- Hot glue gun and glue sticks
- Razorblade
- Scissors

Procedure

1. Using the razorblade, make an X in the middle of one of the lids. Pop this out and fold back the flaps. Cut one of the flaps off to facilitate movement of adults out of bottom chamber.

2. Cut out the middle of the other lid, leaving only the lip that attaches to the container. Do this by making a slit with the razorblade, then finishing cutting with the scissors.

3. Heat up the hot glue gun and glue the two lids together top to top. Run a bead of glue all the way around the outside edges.

4. Cut the bottoms off of two of the plastic tubs using the razorblade.

5. Place one of the bottomless tubs upside down and place the tulle on top of the opening.

6. Press the other bottomless tub upside down on top of the other tub, securing the tulle between the two tubs.

7. Assemble the finished mosquito breeder by snapping the remaining tub and the tulle part into the two sides of the lid (Figure B-1).

Each finished mosquito breeder can hold about 500 mL of water in the bottom container. A cotton ball soaked in sugar solution can be placed on top of the tulle screen to provide food for the adults. Collecting adults from the container is accomplished by placing a lid with a hole punched in the middle over the top of the screening. Introduce a tube through the hole and pump CO_2 into the container for approximately 1 min, or until all mosquitoes are knocked down. The mosquitoes can be collected with forceps. Not all adult mosquitoes will fly up into the upper part of the breeder, so the lid will have to be removed to collect those that remain below. The approximate cost of each mosquito breeder constructed as above is 41 cents.

Figure B-1. Finished mosquito breeder

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BIOGRAPHICAL SKETCH

Tracy Conklin was born on April 17, 1982 in West Palm Beach, Florida. As an only child, she entertained herself by catching insects, lizards, and fish in her subtropical backyard. She examined her first mosquito larva under her mother's microscope at 8 years old. Little did she know that she would spend 3 years of her life doing the same in college. After spending 2 years of her undergraduate career studying British literature, Tracy gave in to her love for insects and changed her major to entomology. As an undergraduate, she worked for Dr. Drion Boucias in the insect pathology laboratory, and after graduating, decided to continue looking at mosquitoes under the microscope.