

Effects and tissue pathology of an unidentified microsporidium
from the convergent lady beetle, *Hippodamia convergens* Guérin-Ménéville
(Coleoptera: Coccinellidae), on three non-target coccinellids

By
Taro Saito

A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Masters in Applied Science

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Abstract

A microsporidium from *H. convergens* was transmitted horizontally to three non-target coccinellids (*Adalia bipunctata* L., *Coccinella septempunctata* L. and *Harmonia axyridis* Pallas) under laboratory conditions. The effects of microsporidiosis on larval development, larval mortality, sex ratio, fecundity and adult longevity were examined. For all beetle species, larval development was prolonged as a result of infection. Fecundity and longevity was adversely affected only for the natural host, *H. convergens*. Spore counts were similar for *A. bipunctata* and *H. convergens* but fewer spores were observed in *C. septempunctata* and *H. axyridis*. Vertical transmission of the pathogen was observed in all host species. Three eugregarines were found and described from two adult *A. bipunctata*. In *H. convergens*, the microsporidium infected muscles surrounding the midgut and within the fat body, Malpighian tubules, pyloric valve epithelium, colon epithelium, nerves, muscles, connective tissues, and ovaries. Tissue pathology differed for *C. septempunctata* and *H. axyridis*.

September 29, 2008

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Introduction

The era of synthetic insecticides began in the middle of the 20th century. Although some scientists and agriculturists believed that pests were finally under control, others began to realize that there were severe adverse effects associated with the use of chemical insecticides. In 1962, Rachel Carson reported the most famous example in her book entitled *Silent Spring*. Carson was first to make a connection between the overuse of dichloro-diphenyl-trichloroethane (DDT) and its ecological impacts: the decline of certain bird populations, health problems in workers who handled DDT, the discovery of DDT in the milk of mothers who were exposed to the chemical, liver cancers in exposed fish, destruction of beneficial insects, and the selection of DDT-resistant insects (Carson, 2002). Her work prompted the search for safer and more environmentally friendly ways to control pest insects. Biological pest control developed significantly after World War II, although the idea itself was not novel.

Biological control is an important pest management method, whereby predatory arthropods, parasitoids and pathogenic organisms are employed to reduce pest populations (Hagler, 2000; Johansen, 1985). There are three types of biological control: (1) classical biological control (exotic natural enemies are imported for controlling introduced pests); (2) augmentative biological control (the release of large numbers of reared or field-collected natural enemies); and (3) conservation biological control (various cultural techniques are used to conserve natural enemies that are already present in a system) (Koch, 2003).

One of the earliest and most dramatic success stories regarding classical biological control took place in California in 1888. Cottony cushion scale, *Icerya*

purchasi Maskell were accidentally introduced into the United States from Australia and heavy infestations were ruining citrus orchards in California (Caltagirone and Douth, 1989). Foreign exploration resulted in the introduction of *Rodolia cardinalis* L. (Mulsant), a lady beetle and natural enemy for cottony cushion scale in Australia, and the pest was almost completely eradicated from California orchards within a few years. This success was recorded before the introduction of chemical insecticides; therefore, the use of biological methods for pest control was reassessed once the ecological impacts of chemical insecticides were realized.

Since the success of *R. cardinalis* against the cottony cushion scale, predaceous coccinellids have been associated with biological pest control more often than other predatory organisms. Coccinellids are important natural enemies of several prominent pest insects such as whitefly, aphids, mealy bugs, scales, and mites (Obrycki and Kring, 1998); therefore, several coccinellid species have been introduced for biological pest control in North America. As a result, North America is currently home to 18 coccinellid species (of 179 species intentionally introduced) that are native to Europe and Asia (Cormier *et al.*, 2000; Obrycki and Kring, 1998). *Coccinella septempunctata* L. and *Harmonia axyridis* (Pallas) are good examples of introduced species that have become established in North America. Colonization of these beetles has been reported since the 1970's and 1990's, respectively (Obrycki *et al.*, 2000).

Despite good intentions, few introduced species succeed as biological control agents. Many studies report native coccinellid species being out-competed by their introduced cousins (Cormier *et al.*, 2000; Elliot *et al.*, 1996; Koch, 2003; Obrycki *et al.*, 2000; Turnock *et al.*, 2003). A 1998 survey of coccinellids in Cape Breton revealed that

the total number of non-native species was 20 times greater than those that were native. The authors concluded that a serious decline in native coccinellids has occurred over the past decade and an increase in competition for food by introduced beetle species was likely the cause (Cormier *et al.*, 2000). Koch (2003) summarized the non-target effects caused by *H. axyridis* on other coccinellid species as: (1) intra guild predation whereby *H. axyridis* shows a greater ability to utilize other coccinellid species as its food source; and (2) resource competition, in which *H. axyridis* is favored due to its voraciousness, high fecundity and fertility. *H. axyridis* also impacts humans and human activities. All lady beetles produce alkaloids and some persons experience allergic reactions when they are bitten by *H. axyridis* or when large populations of beetles overwinter inside houses (Koch, 2003).

In North America, convergent lady beetles, *Hippodamia convergens* Guérin-Méneville, are often purchased for aphid control in biological control programs. *H. convergens* adults are collected annually from their overwintering sites in California and are then sold to commercial growers and home gardeners (Dreistadt and Flint, 1996). Studies have shown *H. convergens* to be ineffective for aphid suppression because beetles have a tendency to disperse once they are released in the field (Kieckhefer and Olson, 1974; Dreistadt and Flint, 1996; Obrycki and Kring, 1998). Despite these reports, *H. convergens* remains a popular choice for biological control because these beetles are easy to collect and distribute and they may provide effective pest control in enclosed areas such as greenhouses. However, several natural enemies have been associated with *H. convergens* and these may be inadvertently imported and released when beetles are used in biological control programs (Bjørnson, 2008).

Three species of microsporidia (Phylum Microspora, obligate, intercellular, spore-forming, protozoan parasites) have been reported from field-collected coccinellids. In 1959, *Nosema hippodamiae* Lipa and Steinhaus was described from the midgut and fat body of *H. convergens* (Lipa and Steinhaus, 1959). Afterward, Sluss (1968) reported an unidentified microsporidium in *H. convergens* and its endoparasitoid (parasitic wasp), *Dinocampus coccinellae* Shrank; however, it is not clear if this pathogen is *N. hippodamiae*. Two additional species of microsporidia have been reported from other field-collected coccinellids. *N. tracheophila* Cali and Briggs infects the tracheal epithelium, hemocytes and connective tissues of *C. septempunctata* (Cali and Briggs, 1967) and *N. coccinellae* Lipa is known to infect the midgut epithelium, Malpighian tubules, gonads, nerves and muscle tissues of *C. septempunctata*, *H. tredecimpunctata* L. and *Myrrha octodecimguttata* L. (Lipa, 1968a); in addition, this same microsporidium was later reported from three other coccinellid hosts: *C. quinquepunctata* L., *Adalia bipunctata* L., and *Exochomus quadripustulatus* L. (Lipa *et al.*, 1975). In 2004, an unidentified microsporidium was found in *H. convergens* that were purchased from a commercial insectary (Saito and Bjørnson, 2006).

Microsporidia produce environmentally-resistant, infective spores that are protected by thick walls of protein and chitin (Franzen, 2004; Keeling and McFadden, 1998). The spore contains a polar tube that is coiled around the nucleus, which is discharged through the thin anterior end of the spore to penetrate the host cell. An infective sporoplasm is then squeezed through the polar tube into the cell cytoplasm (Franzen, 2004; Keeling and McFadden, 1998). Microsporidia lack mitochondria. Once they are within the host cell, energy is diverted from the host for pathogen development.

The sporoplasm matures into a meront which undergoes asexual reproduction, leading to the production of 50 to several hundred meronts. Meronts develop into sporonts, then into sporoblasts that eventually become mature spores. Spores are released when the host cell lyses (Franzen, 2004).

Until recently, microsporidia were thought to be host specific under natural conditions (Kluge and Caldwell, 1992), which may explain why *H. convergens* has been used for aphid control even after *N. hippodamiae* was discovered in 1959. Host ranges of microsporidia are broader than previously thought and characteristics of infection (including tissue pathology) vary depending on host species infected (Canning, 1962; Solter *et al.*, 1997; Solter and Maddox, 1998). According to Solter *et al.* (1997), many insect pathogens exhibit a broader host range when unnatural methods of exposure (direct injection of microsporidian-spore suspensions) are studied under laboratory conditions, rather than when more realistic methods are used. Laboratory conditions may be ideal for parasite transmission because the host often receives a maximum or optimal dose of the pathogen. This kind of exposure does not reflect ecological factors such as the distribution and survival of infective stages in the environment.

In general, horizontal transmission (transmission of parasites from an infected host to previously-uninfected individuals) occurs through oral and body surface portals, but the most common route is through ingestion of contaminated food (Andreadis, 1987; Tanada and Kaya, 1993). Vertical transmission (one generation to the next) occurs by the ovarial portal (Tanada and Kaya, 1993). Few studies have explored horizontal transmission of microsporidia in coccinellids under laboratory conditions. Cali and Briggs (1967) were able to infect adult *C. septempunctata* with *N. tracheophila* by

directly feeding adult beetles a mixture of honey and spores, but the authors did not investigate host specificity of the pathogen. A previous study (Saito and Bjørnson, 2006) examined horizontal transmission of an unidentified microsporidium from *H. convergens* to three field-collected coccinellids from Nova Scotia (*C. septempunctata*, *C. trifasciata perplexa* Mulsant, and *H. axyridis*). Egg cannibalism and egg predation were investigated as the means of horizontal transmission by allowing first-instar larvae to consume microsporidia-infected eggs. This is likely to occur under natural conditions because many predaceous coccinellids are cannibalistic (Agarwala, 1991, 1998; Agarwala and Dixon, 1992) and newly-hatched larvae often feed on other sibling larvae or on eggs from the same clutches that fail to hatch. When prey availability is low, beetles consume eggs or younger larvae and these larvae are often vulnerable to older ones. In this case, the microsporidium was transmitted with 100% efficacy for all beetle species examined. Mean spore counts were similar for *C. trifasciata perplexa* (a native species) and *H. convergens*, suggesting that the former was a suitable host for the microsporidium. Lighter infections observed in *C. septempunctata* and *H. axyridis* suggest that these introduced beetles may be less suitable for the microsporidium to complete its development. Further study is needed to determine if the pathogen is transmitted vertically among individuals of each species. Successful vertical transmission would suggest that the microsporidium is capable of becoming established within coccinellid communities.

Microsporidia are known to cause chronic, debilitating disease that lowers host fitness (Tanada and Kaya, 1993). Symptoms include abnormal feeding, irregular growth, delayed larval development, incomplete metamorphosis, deformed pupae and adults,

decreased fecundity and longevity, or death (Kluge and Caldwell, 1992). Symptoms may vary depending on the tissues that are infected. Larval development is significantly longer for microsporidia-infected *H. convergens*, *C. septempunctata*, *C. trifasciata perplexa*, and *H. axyridis* than for their uninfected cohorts but larval mortality is not affected (Saito and Bjørnson, 2006).

Although the microsporidium significantly reduces adult longevity and fecundity of *H. convergens* (Joudrey and Bjørnson, 2007), the effects of this pathogen on non-target coccinellids have not been investigated. Therefore, the objectives of this study are: (1) to examine the effects of the unidentified microsporidium on adult longevity and fecundity of three non-target coccinellids (*Adalia bipunctata* L., *C. septempunctata*, and *H. axyridis*), (2) to determine if vertical transmission of the microsporidium occurs among these hosts, and (3) to examine the tissues that are infected by the microsporidium in *H. convergens* and the non-target species.

For all host species examined, longevity and fecundity are expected to be lower in beetles from the treatment groups (those fed microsporidia-infected eggs as first-instar larvae) when compared to those in the control groups. The severity of infection (in terms of spore counts) is dependent on the host species when the pathogen was horizontally transmitted through microsporidia-infected eggs (Saito and Bjørnson, 2006); therefore, the magnitude of the effects of the pathogen on host fitness is also likely to vary with the host. Earlier descriptions of microsporidia from coccinellids include a list of infected tissues and these differ depending on the microsporidium that is described (Lipa & Steinhaus, 1959; Cali & Briggs, 1967; Lipa 1968a). Therefore, an examination of tissue pathology may help identify the pathogen and explain differences in fitness or vertical

transmission (if any variations are observed). Tissue pathology should vary among the host species examined. Infection of specific tissues may be diagnostic for the pathogen and infection of the ovaries may be necessary for vertical transmission of the pathogen.

Chapter 1:

Effects of a microsporidium from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), on three non-target coccinellids

1.1. Introduction

In North America, convergent lady beetles, *Hippodamia convergens* Guérin-Méneville, are collected each year from their overwintering sites in California. These beetles are packaged and sold to commercial growers and home gardeners for aphid control. Several natural enemies are associated with field-collected *H. convergens* (Bjørnson, 2008) and because beetles are not mass-reared or placed under quarantine prior to use, their natural enemies are inadvertently imported and released when beetles are used for biological control.

Three species of microsporidia have been described from coccinellids. In 1959, *Nosema hippodamiae* was described from field-collected *H. convergens* from California (Lipa and Steinhaus, 1959). Microsporidia are thought to be host specific under natural conditions (Kluge and Caldwell, 1992) and this may explain why *H. convergens* were collected and distributed for aphid control even after *N. hippodamiae* was discovered in 1959. *N. tracheophila* was later described from laboratory-infected *Coccinella septempunctata* L. (Cali and Briggs, 1967) and *N. coccinellae* Lipa was found infecting several coccinellids that were examined from their overwintering sites (Lipa, 1968a; Lipa *et al.*, 1975).

In a recent study, an unidentified microsporidium from *H. convergens* was transmitted to three non-target coccinellids (*C. septempunctata*, *C. trifasciata perplexa*

Mulsant and *Harmonia axyridis* Pallas) when larvae were fed microsporidia-infected *H. convergens* eggs under laboratory conditions (Saito and Bjørnson, 2006). Successful pathogen transmission from *H. convergens* (the natural host) to other coccinellids raises questions regarding host specificity and the effects of this pathogen on non-target coccinellids. The objective of this study was to examine the effects of the microsporidium from *H. convergens* on life history characteristics (larval development and mortality, adult longevity and fecundity, sex ratio) of three non-target coccinellids: *Adalia bipunctata* L., *C. septempunctata*, and *H. axyridis*. Vertical transmission of the pathogen was also investigated.

A. bipunctata was chosen for this study because it is native to North America and is commercially available for biological control in both North America and Europe. Although *C. trifasciata perplexa* was the subject of a previous study (Saito and Bjørnson, 2006), this native coccinellid was difficult to rear and field-collected specimens were in short supply. Both *C. septempunctata* and *H. axyridis* are introduced species: the former was introduced into North America from Europe, whereas the latter is from Asia. Colonization of these two beetle species has been reported since the 1970's and 1990's, respectively (Obrycki *et al.*, 2000) and they appear to be responsible for a recent and serious decline of indigenous coccinellid species (Cormier *et al.*, 2000; Elliot *et al.*, 1996; Koch, 2003; Obrycki *et al.*, 2000; Turnock *et al.*, 2003).

1.2. Materials and Methods

Uninfected and microsporidia-infected *H. convergens* were obtained from a shipment of beetles that were purchased from a commercial insectary in July 2004. Beetles were reared in 120 mL clear, polyethylene cups (Canemco-Marivac Inc, QC) in environmental chambers (16L:8 D; 25±1°C:20±1°C). Each rearing cup had a 2.2 cm diameter hole in its side that was covered with a fine mesh screen (80µm, Bioquip, CA). The screen allowed air to circulate but prevented beetles from escaping. Cups were washed, soaked in a 10% bleach solution (10 min), rinsed, and then air-dried before use. A piece of filter paper (55 mm diameter) was used to line the inside of each lid. Beetles were supplied distilled water as needed through a moistened cotton roll (Crosstex International, NY).

Beetles were maintained on green peach aphids (*Myzus persicae* Sulzer) that were reared under controlled conditions (16L:8D; 25±1°C:20±1°C). Aphid colonies were reared on nasturtium (*Tropaeolum minus* L., Dwarf Jewel Mix, Stokes Seeds Ltd., ON) that were grown from seed (16L:8D; 25±1°C:20±1°C). When beetles were being maintained (when they were not used in trials or used to rear offspring), they were fed an artificial diet that consisted of Lacewing & Ladybug Food (20 mL, Planet Natural, MT), honey (20 mL), and distilled water (2 mL). This diet was nutritionally sufficient to keep beetles alive; however, it proved insufficient as a sole source of food for oviposition. Fresh diet was kept under refrigeration and was supplied to beetles as needed. Prior to feeding, a small amount of diet was softened at room temperature and sterilized spatulas were used to spread the diet on the inside wall of each rearing cup at the edge of the screen-covered hole.

Laboratory colonies of *A. bipunctata*, *C. septempunctata*, and *H. axyridis* were established from specimens collected on Saint Mary's University Campus. The progeny of these individuals were used to establish microsporidia-free beetle colonies that were maintained and reared in the same manner as was *H. convergens*. Eggs and larvae from these colonies were examined on a routine basis to ensure that individuals were free of microsporidia.

1.2.1 Horizontal transmission

During the experimental trials, all larval and adult coccinellids were fed an *ad libitum* diet of green peach aphids that was augmented with bird cherry-oat aphids (*Rhopalosiphum padi* L.; purchased from a commercial insectary) and rose aphids (*Macrosiphum rosae* L.; collected from shrub roses on the Saint Mary's University campus). Instruments used for transferring eggs, handling larvae, and feeding (feather-weight forceps, spatulas and fine brushes) were dipped in 70% ethanol (1 min), then rinsed in distilled water after each use to prevent contamination. Procedures (beetle feeding and observation) were carried out in a biological safety cabinet (Baker Company, SterilGARD III Advanced, SG403A). Control (uninfected) groups were fed before treatment (microsporidia-infected) groups. Microsporidian spores are known to lose their viability when they are subjected to UV light or when they become desiccated (Maddox, 1977; Kelly and Anthony, 1979; Whitlock and Johnson, 1990); therefore, the interior surfaces of the cabinet were disinfected with 70% ethanol, followed by exposure to a germicidal UV light (Philips TUV36T5/SP 40W, 253.7 nm, 60 cm above working surface) for 15 min before and after each daily observation period.

Larvae and eggs used to set up the trial were confirmed as either microsporidia-free or microsporidia-infected by examining other eggs and larvae that were produced by the same parent female. After enough eggs were collected for setting up the trial, parent females were also examined. Smear preparations of eggs, larvae and parent beetles were fixed in methanol, stained in 10% buffered Giemsa and examined for microsporidian spores by light microscopy. Throughout the trial, this staining methodology was used to prepare and examine individuals for microsporidian spores.

H. convergens was used as a reference species to verify that the microsporidian spores within infected eggs were viable and able to infect the non-target coccinellids species. For *H. convergens*, 14 uninfected mating pairs were used for rearing larvae that were used in the trial and 12 microsporidia-infected mating pairs were used to produce infected eggs that were fed to larvae from all treatment groups. Twelve uninfected mating pairs were used to rear *A. bipunctata*, *C. septempunctata*, and *H. axyridis* larvae.

For each species, one uninfected *H. convergens* egg was fed to each 1-day-old uninfected larva ($n = 50$ larvae), which served as a reference (control). Larval age was an important consideration because larvae finish consuming their egg shells and start searching for food when they are about 1-day-old. Conversely, one microsporidia-infected *H. convergens* egg was fed to each uninfected 1-day-old larva ($n = 50$ larvae; treatment).

Twenty polyethylene cups (10 control and 10 treatment) were prepared for each species on alternate days. This procedure was repeated five times until 100 uninfected larvae ($n = 50$ control and $n = 50$ treatment) of each species were set up. Larvae of all species were reared individually. A sterile cotton roll was moistened with distilled water

and placed in each cup. One *H. convergens* egg (less than 24 h old) was transferred onto a piece of sterilized filter paper (6 mm diameter) that was pre-moistened with distilled water. The disk was placed carefully at the bottom of a rearing cup and one 1-day-old larva was transferred onto the paper so that it was adjacent to the egg. Larvae that did not eat the egg after 24 h had lapsed and those that died prior to their first molt were discarded. Larvae were then reared on an *ad libitum* diet of aphids and distilled water that were provided daily until the larvae completed development and emerged as adults. Larvae that failed to complete development were smeared and examined for microsporidian spores. Presence and absence of spores in all individuals (larvae that failed development, emerged males, emerged females that were employed in the following observations) were used to produce percent horizontal transmission.

1.2.2 Effects on larval development and mortality

For each species, a one-tailed t-test (control < treatment, $\alpha = 0.05$) was used to determine significance in larval development time (from first instar to adult emergence) between individuals from control and treatment groups. Adults were sexed following eclosion and this allowed male and female larvae to be differentiated. Larval development data were reanalyzed to determine the effect of the microsporidium on the development time of male and female larvae (uninfected male vs. uninfected female larvae; infected male vs. infected female larvae). Data were tested for normality (Shapiro-Wilk W test) and only individuals that completed their development and emerged as adults were included in the analysis. A χ^2 test ($\alpha = 0.05$) was used to analyze differences in larval mortality between control and treatment groups. Larvae that did not

eat the egg after 24 h and those that died prior to their first molt were excluded from the analysis.

1.2.3 Sex Ratio

Adults were sexed upon emergence and a χ^2 test was used to test significance ($\alpha = 0.05$) in sex ratios between control and treatment groups for each beetle species. After all beetles were sexed, newly-emerged female beetles were mated and used in 90-day fecundity and longevity trials. Male beetles that emerged were smeared and examined for microsporidian spores.

1.2.4 Effects on adult fecundity and longevity

Newly-emerged, virgin females were placed individually in cups and provided artificial diet and distilled water for four days. Females were fed aphids on the fifth day following emergence and they were mated on the sixth day. In the case of *H. convergens*, females were mated with uninfected males that were obtained from a shipment of beetles. For all other beetle species, virgin females were mated with uninfected and genetically-distant males that originated from uninfected parents. Mating pairs were provided distilled water and an *ad libitum* diet of aphids. After mating, male beetles were smeared and examined for microsporidian spores. Mating periods were longer for some beetle species (24 h for *A. bipunctata*, 48 h for *H. convergens* and *H. axyridis*, and 72 h for *C. septempunctata*) to ensure that viable eggs were produced.

Mated females were provided distilled water and aphids daily for the duration of the trial and artificial diet was also provided as needed. Eggs were removed and counted

daily. All of the females were smeared upon death or at the end of the trial and examined for microsporidian spores. Age-specific oviposition curves were constructed for each species and a one-tailed t-test (control > treatment, $\alpha = 0.05$) was used to determine significance in fecundity (grand means) between individuals from control and treatment groups. Data were tested for normality (Shapiro-Wilk W test) and non-normal data (*H. convergens* & *C. septempunctata*) were power-transformed. A χ^2 test ($\alpha = 0.05$) was used to test for significance in adult longevity by comparing the ratios of live and dead beetles between control and treatment groups at the end of the trial (90 days post emergence).

1.2.5 Spore counts

Spore counts were used to determine the relative suitability of each beetle species as host for the microsporidium. For each species, smear preparations were made from randomly-chosen females from the treatment groups ($n = 4$ per species). Heads and pronota were removed before the remainder of the body was smeared and examined for microsporidian spores. The mean ages of the examined females were 77.0, 72.0, 73.5, and 90.0 days (post eclosion) for *H. convergens*, *A. bipunctata*, *C. septempunctata* and *H. axyridis*, respectively. Twenty five areas (each area: $100 \mu\text{m}^2$) were examined from random sites on each slide (total $n = 100$ counts/species, 1000X magnification). The mean number of spores per area was calculated for each species and spore counts from non-target species were compared to those of *H. convergens*. Raw spore count data were log transformed and re-assessed for normality (Shapiro-Wilk W test). Transformed data were analyzed for significance with a one-way ANOVA and Dunnett's multiple comparison test ($\alpha = 0.05$) (Statistix 8, 2003).

1.2.6 Vertical transmission

For each beetle species, eggs and larvae produced by females of the treatment groups ($n = 4$ for each species) were examined for microsporidian spores over the lifetime of each female. Microsporidian spores were easier to detect in first-instar beetle larvae than in eggs; therefore, eggs were isolated daily and allowed to hatch (3-4 days). A maximum of 10 randomly-selected larvae (or 10 eggs if they did not hatch) from each female were smeared on each day of the trial, and examined for microsporidian spores. Presence or absence of spores was used to determine daily percent vertical transmission (number of larvae that contained spores / total number of larvae examined) and to construct vertical transmission curves for each beetle species.

1.3. Results

1.3.1 Horizontal transmission

Microsporidian spores were not detected in smear preparations made from any of the parent males that were mated with female beetles during the trial. Female *H. convergens* that were used to produce uninfected eggs (that were fed to 1-day-old control larvae or used as first-instar larvae for the trial) were also free of microsporidian spores. To ensure that a low infection would not be overlooked, additional eggs and cohort larvae were also examined but spores were not observed in any of the smear preparations. Conversely, spores were detected in all *H. convergens* females that were used to produce eggs that served as food for 1-day-old treatment larvae. All additional eggs and larvae that were examined from these microsporidia-infected females were infected with spores.

At the end of the 90-day trial, microsporidian spores were not detected in *H. convergens*, *A. bipunctata*, *C. septempunctata*, or *H. axyridis* from the control groups including males, females, and larvae that failed to complete development ($n = 47, 42, 45,$ and $44,$ respectively). Microsporidian spores were detected in the majority of smear preparations of individuals from the treatment groups (*H. convergens*, 40 infected/46 total = 87 % transmission; *A. bipunctata*, 35/38 = 92.1 %; *C. septempunctata*, 40/46 = 87 %; *H. axyridis*, 38/42 = 90.5 %).

1.3.2 Effects on larval development and mortality

For all species examined, treatment larvae took significantly longer to develop than did control larvae (*H. convergens*, $t = -4.55$, $df = 81$, $P = 0.001$; *A. bipunctata*, $t = -3.78$, $df = 58$, $P = 0.001$; *C. septempunctata*, $t = -3.8$, $df = 61$, $P = 0.001$; *H. axyridis*, $t = -5.54$, $df = 71$, $P = 0.001$) (Table 1.1). There was no significant difference in larval development based on sex (uninfected male vs. uninfected female larvae; infected male vs. infected female larvae; $P > 0.05$).

Significant differences in larval mortality were not observed. Results from *C. septempunctata* were valid ($\chi^2 = 0.24$, $df = 1$, $P = 0.62$); however, χ^2 results for the other beetle species were probably invalid since two cells (both control and treatment) had expected counts less than five (*H. convergens*, $\chi^2 = 0.56$, $df = 1$, $P = 0.45$; *A. bipunctata*, $\chi^2 = 3.75$, $df = 1$, $P = 0.05$; *H. axyridis*, $\chi^2 = 0.55$, $df = 1$, $P = 0.46$) (Table 1.1).

1.3.3 Sex Ratio

Sex ratios of emerged adults were about 1:1 ♀: ♂ (Table 1.2). Significant differences were not observed among individuals of the control and treatment groups (*H. convergens*, $\chi^2 = 0.82$, $df = 1$, $P = 0.37$; *A. bipunctata*, $\chi^2 = 0.07$, $df = 1$, $P = 0.79$; *C. septempunctata*, $\chi^2 = 0.001$, $df = 1$, $P = 0.99$ *H. axyridis*, $\chi^2 = 0.15$, $df = 1$, $P = 0.7$).

1.3.4 Effects on adult fecundity and longevity

Females from the treatment groups that were not infected with microsporidia at the end of the 90-day trial were excluded from the analysis (3 *H. convergens*, 3 *C. septempunctata* and 1 *H. axyridis*). Unidentified eugregarines were observed in a single *A. bipunctata* female (control) and one male (treatment). Although eugregarines are considered to be weak pathogens that cause little damage to host tissues (Tanada and Kaya, 1993), the infected female was also excluded from the analysis. Eugregarine trophozoites were described according to Clopton (2004).

Gregarine A ($n = 22$ trophozoites; Table 1.3) was observed in the *A. bipunctata* male. No associations (syzygy) were observed. Trophozoites stained lightly and were granular in appearance. The protomerite appeared broadly ovoid, being wider than long. The deuteromerite was dolioform to eliptoid, and a single spherical nucleus was visible in more than half of specimens (mean diameter, $8.4 \mu\text{m}$, $n = 12$). Gregarine B ($n = 34$) was observed in the *A. bipunctata* female. No associations were observed. Trophozoites of Gregarine B were similar in size to those of Gregarine A but the former had a distinctly smaller and more darkly stained deuteromerite. The protomerite was ovoid and the deuteromerite was panduriform. About one third of the deuteromerites contained a single

spherical nucleus (mean diameter, 7.8 μm , $n = 11$). Gregarine C ($n = 13$) was also observed in the *A. bipunctata* female. Trophozoites of Gregarine C were much larger than those of Gregarines A and B and were nearly translucent and granular in appearance (Table 1.3). Syzygy was observed as bi-associations of primitives with satellite trophozoites of similar dimensions. The protomerite was shallowly ovoid, being wider than long. The majority of deutomerites were dolioform but some were pyriform or obpyriform, containing a single spherical nucleus (mean diameter, 11.9 μm , $n = 9$).

Age-specific oviposition curves (mean eggs / day) were constructed for each beetle species (Fig. 1.1). During the 90-day trial, *H. convergens* females from the treatment group produced significantly fewer eggs than did those of the control group ($t = -2.0$, $df = 34$, $P = 0.03$). However, no significant differences in fecundity were observed between control and treatment *A. bipunctata*, *C. septempunctata*, and *H. axyridis* females ($t = -0.42$, $df = 33$, $P = 0.66$; $t = -0.63$, $df = 30$, $P = 0.27$; $t = -1.27$, $df = 35$, $P = 0.89$, respectively) (Table 1.2).

Percent mortality for all beetle species (Table 1.2) was calculated at the end of the trial. *H. convergens* females of the control group lived significantly longer than did females from the treatment group ($\chi^2 = 4.53$, $df = 1$, $P = 0.03$). Significant differences were not observed for *A. bipunctata* ($\chi^2 = 0.54$, $df = 1$, $P = 0.46$) and *C. septempunctata* ($\chi^2 = 0.05$, $df = 1$, $P = 0.83$). In the case of *H. axyridis* ($\chi^2 = 0.26$, $df = 1$, $P = 0.61$), two cells (for both the control and treatment) had expected numbers that were less than five and this would likely render the χ^2 approximation invalid.

1.3.5 Spore counts

Mean spore counts were used to determine the overall suitability of each beetle species as a host for the microsporidium (Table 1.2). Mean spore counts for *A. bipunctata* (134.4 ± 8.8 spores / $100 \mu\text{m}^2$) did not differ significantly from *H. convergens* (101.5 ± 5.6), the natural host of the microsporidium; however, spore counts for *C. septempunctata* (32.4 ± 3.1) and *H. axyridis* (24.8 ± 3.3) differed significantly ($F = 176$, $df = 3$, $P < 0.0001$).

1.3.6 Vertical transmission

Percent vertical transmission data were acquired by examining eggs that were produced by randomly chosen females ($n = 4$ for each species) (Fig. 1.2). In the case of *H. convergens*, vertical transmission of the microsporidium was detected in 70% of the eggs that were produced on the first day of oviposition and almost 100% transmission was observed about 9 days after oviposition had begun. Microsporidian spores were also detected in about 37% of the eggs produced by *A. bipunctata* on the first day of oviposition. The number of eggs that contained spores increased until 26 days later when all were infected. For *C. septempunctata*, microsporidian spores were detected in 12.5% of the eggs that were produced on the first day and in more than 80% of the eggs that were produced seven days later. In the case of *H. axyridis*, microsporidian spores were initially detected in only a few eggs (2.5 %) but eventually spores were detected in all of the eggs that were produced (100%).

1.4. Discussion

Despite rigorous efforts to ensure that the non-target beetles used in the trials were free of microsporidia and other pathogens, three unidentified eugregarines were found in two adult *A. bipunctata*. Based on trophozoite dimensions, Gregarine A (34.1-64.9 x 16.5-46.0 μm , TL x WD) were similar in size to *Gregarina katherina* Watson (45-70 x 20-34 μm ; Watson, 1915), described from *Coccinella novemnotata* Herbst, *C. septempunctata bruckii* Mulsant, *C. californica* Mann and *C. trifasciata* L. (Hoshide, 1951; Lipa, 1968b). Trophozoites of Gregarine B (24.4-44.9 x 9.3-24.5 μm) have similar dimensions to those of Gregarine A but their morphology was distinct. Although *G. ruszkowskii* is reported from *A. bipunctata* (Lipa *et al.*, 1975), both Gregarine A and B were too small to be *G. ruszkowskii* (65-92 x 30-83 μm , Lipa, 1967). Gregarine C (primate, 62.2-137.2 x 20.9-77.6 μm) was similar in size to *G. barbarara* Watson, previously described from *A. bipunctata* (60-100 x 40-60 μm ; Watson, 1916).

Horizontal transmission was lower during this study than was reported previously by Saito and Bjørnson (2006) and this may be due to a difference in spore dose. One microsporidia-infected *H. convergens* egg was fed to each treatment larva during this study but as many as three infected eggs (and presumably a larger spore dose) were eaten by beetle larvae in the previous study.

Larval development for microsporidia-infected larvae (treatment) was delayed by about one day when compared to uninfected larvae (control). Larval mortality, however, was not affected. These findings are consistent with those reported in a previous study (Saito and Bjørnson, 2006); however, *H. convergens*, *C. septempunctata* and *H. axyridis* larvae took longer to develop during this study than they did previously. Differences in

development times may be attributed to different rearing temperatures that were used in the two studies. Larval mortality was observed for most species (both control and treatment) but larval mortality was relatively high for *C. septempunctata* larvae from both the control and treatment groups. High larval mortality for *C. septempunctata* larvae was also observed previously (Saito and Bjørnson, 2006). Coccinellid eggs are coated with species-specific alkaloids that may be toxic to other species (Cottrell, 2004, 2005; Omkar *et al.*, 2004; Sato and Dixon, 2004) and this may explain the high mortality observed for *C. septempunctata* larvae. Larval mortality may also have been caused, in part, by the cherry-oat aphids (*Rhopalosiphum padi*) that were used for food in this study because this aphid species is considered to provide relatively low quality food for *C. septempunctata* (Hauge *et al.*, 1998).

Results from this study suggest that *A. bipunctata* (a native species) was a suitable host for the microsporidium but low spore counts in *C. septempunctata* and *H. axyridis* (both introduced, highly invasive species) suggest that these two host species were less favorable for the reproductive success of the pathogen. Nevertheless, the microsporidium appeared to reproduce successfully during the adult life span in three host species. For *H. convergens*, newly emerged adults had 12.8 ± 1.16 spores/100 μm^2 (Saito and Bjornson, 2006), whereas 101.5 ± 5.6 spores/100 μm^2 were observed in older adults from this study (mean age: 77 days post emergence). Similar comparisons may be made for *C. septempunctata* and *H. axyridis*. Newly emerged adults had 7.5 ± 0.65 and 0.8 ± 0.11 spores/100 μm^2 , respectively (Saito and Bjornson, 2006) but older adults of the same species had 32.4 ± 3.1 (mean age: 74 days post emergence) and 24.8 ± 3.3 spores/100 μm^2 (mean age: 90 days post emergence), respectively.

Although spore count data demonstrated that successful reproduction of the pathogen took place within the non-target hosts, it is difficult to explain why the non-target hosts were not adversely affected. Prolonged larval development was observed for all host species examined and this is typical of microsporidiosis. Furthermore, the consumption of one microsporidia-infected egg by first-instar larvae was sufficient to cause adverse effects in adult fecundity and longevity in the natural host (*H. convergens*).

The pathogen did not affect adult fecundity and longevity of the three non-target coccinellids but this may be explained, in part, by the conditions that beetles were subjected to during the trial. Adult beetles had constant access to clean water and were provided an *ad libitum* diet of aphids and artificial diet (pollen, yeast and honey). When microsporidia-infected beetles are fed artificial diet, the energy gained from the diet may compensate for the loss of energy caused by the microsporidium because honey and sugar provide energy (Hagen, 1962; Thompson, 1999) and pollen and fungi provide beetles with alternative sources of protein (Hagen, 1962). For example, *Coleomegilla maculata* (DeGeer) larvae are able to complete development when fed only pollen (Hodek, 1996) and larval survival, adult fecundity and percent egg hatch of *A. bipunctata* are improved when beetles are fed frozen pollen and *Ephestia kuehniella* Zeller eggs rather than when they are fed moth eggs alone (De Clercq *et al.*, 2005). It has also been suggested that many polyphagous coccinellids, *A. bipunctata* and *H. axyridis* for example, employ a mixed feeding habit to select for a favorable balance of nutrients from various food sources, including plant materials (Hodek, 1996). Food quality appeared to increase beetle fecundity, whether or not individuals were ultimately infected with the microsporidium. Both uninfected and microsporidia-infected *H. convergens* fed aphids

and artificial diet during this study laid more eggs (grand mean of 1458 and 1199 eggs, respectively) than ones fed aphids only (*M. persicae*) (928 and 545 eggs, respectively; Joudrey and Bjørnson, 2007). These results suggest that the artificial diet was responsible for the increase in fecundity that was observed for *H. convergens*.

Although differences in prey species and environment must be considered, other coccinellid species examined in this study also performed better than they did during previous studies. Uninfected and microsporidia-infected *A. bipunctata* from this study, which were fed both aphids and artificial diet, produced an average of 1327 and 1385 eggs, respectively and 52.4% of the control and 40% of the treatment beetles survived until the end of the 90-day trial (Table 1.2). When fed only pea aphids, *Acyrtosiphon pisum* (Harris), *A. bipunctata* produce an average of 809.9 eggs and lived 70.5 days (16L:8D, 22°C; Ueno *et al.* 2004). Similarly, both uninfected and microsporidia-infected *C. septempunctata* from the present study laid more eggs (mean: 2111 and 1900 eggs, respectively) and lived longer than they did during previous studies. In a study conducted by Kawauchi (1985), *C. septempunctata* produce 1660.5 eggs when fed cotton aphids, *Aphis gossypii* Glover (14L:10D, 25°C). When fed cowpea aphids, *Aphis craccivora* Koch, *C. septempunctata* laid 1060.7 eggs and lived about 70 days (14L:10D, 25°C; Omkar *et al.* 2005).

For all species examined, 100 % vertical transmission was eventually observed (Fig. 1.2) and the transmission patterns appeared to reflect the susceptibility of a particular beetle species to the microsporidium. *H. convergens* (natural host, 101 spores /100 μm^2 , 70 % initial vertical transmission that increased to 100 % transmission 9 days after the first eggs were produced) was the best suited host for the pathogen but *A.*

bipunctata (a native species) was the most susceptible non-target host (134 spores/100 μm^2 , 37 % initial transmission that increased to 100% transmission 26 days later). The two introduced coccinellid species seemed to be less susceptible to the microsporidium: *C. septempunctata* (32 spores/100 μm^2 , 12.5% initial transmission that increased to 100% transmission 54 days later) and *H. axyridis* (25 spores/100 μm^2 , 2.5 % initial transmission that increased to 100% transmission 79 days later). Long-term studies on vertical transmission of microsporidia are needed to assess the fate of these pathogens once they are released into new local environments.

Based on the results of this study, the host range of the microsporidium from *H. convergens* overlaps with the host ranges of *Nosema hippodamiae*, *N. coccinellae* and *N. tracheophila* reported in previous studies (Cali and Briggs, 1967; Lipa 1968a; Lipa and Steinhaus, 1959; Lipa *et al.* 1975). Spore sizes of these microsporidia are similar but the geographical locality of these pathogens is distinct: *Nosema hippodamiae* and *N. tracheophila* are reported from the USA, whereas *N. coccinellae* is reported from Europe. *A. bipunctata* (a known host of *N. coccinellae*) are native to North America (Gordon, 1985) but this species may be purchased from Europe and imported to North America for aphid control. Therefore, it is possible for *N. coccinellae* to be transported from Europe to North America if microsporidia-infected *A. bipunctata* are imported for biological control programs. Although *N. coccinellae* is reported only from European populations of *A. bipunctata*, it is possible that the undescribed microsporidium in *H. convergens* originated from native *A. bipunctata* populations.

Microsporidiosis of non-target hosts have been observed in Lepidoptera (Solter *et al.*, 1997; Solter and Maddox, 1998) and one could argue that host range expansion to

non-target host species is unlikely to occur in a natural environment. In our study, however, the pathogen was readily transmitted to non-target coccinellids when they ate one microsporidia-infected *H. convergens* egg. Although effects on fecundity and adult longevity were not observed, larval development was delayed about one day, and percent vertical transmission increased as the hosts aged, especially in the case of *C. septempunctata* and *H. axyridis* (Fig. 1.2).

Intraguild predation (IGP) and cannibalism are common among predaceous coccinellids (Agarwala, 1991, 1998; Agarwala and Dixon, 1992), not only when prey availability is low but also when prey are abundant (Gupta *et al.*, 2006). Eggs and young larvae are especially vulnerable to older, larger ones. Microsporidia-infected larvae take longer to develop and cannibalism of infected larvae by uninfected ones may facilitate horizontal transmission of the microsporidium. The coccinellid species that were studied here overlap in their distributions and habitats in North America (Boiteau *et al.*, 1999; Cormier *et al.*, 2000; Gordon, 1985; LaMana and Miller, 1996; Majka and McCorquodale, 2006; Yasuda *et al.*, 2004), and IGP among these species have been observed and studied (Kajita *et al.*, 2000, 2006; Yasuda *et al.* 2004). Distribution overlap provides an opportunity for the microsporidium from *H. convergens* to expand its distribution and host range once infected *H. convergens* are released.

This study was designed to reduce environmental stresses so that the effects of microsporidiosis on beetle life history characteristics could be observed. However, such stresses may accentuate differences in fitness between uninfected and infected beetles. Adult beetles use energy searching for prey and for reproduction (Dixon, 2000). In their natural environment, beetles must fly and search for patchily distributed prey and

reproduce with limited resources. Climactic conditions may be unfavorable for optimal growth and reproduction. Physical exercise, flight and heat may provide enough stress to decrease disease resistance (Adamo and Parsons, 2006); therefore, microsporidiosis may have a greater impact on the fitness of non-target coccinellids in their natural environment than was observed during this study.

Table 1.1. Developmental time (days) and percent larval mortality for *H. convergens* and three non-target coccinellids infected with a microsporidium under laboratory conditions

	Developmental time (days) ¹			Larval mortality (%)		
	<i>n</i>	Mean ± SE	<i>P</i> -value	<i>n</i>	Mortality (%)	<i>P</i> -value
<i>Hippodamia convergens</i>						
control	47	18.02 ± 0.01		48	2.1	
treatment	37	18.65 ± 0.01	0.001	40	7.5	0.45 ^a
<i>Adalia bipunctata</i>						
control	42	15.79 ± 0.08		42	0	
treatment	32	16.31 ± 0.11	0.001	35	8.6	0.05 ^a
<i>Coccinella septempunctata</i>						
control	34	17.74 ± 0.16		45	24.4	
treatment	32	18.69 ± 0.19	0.001	40	20.0	0.62
<i>Harmonia axyridis</i>						
control	43	18.12 ± 0.11		44	2.3	
treatment	36	19.08 ± 0.13	0.001	38	5.3	0.46 ^a

¹ Development time measured between first instar and adult eclosion. ^a Two cells (both control and treatment) had expected values less than five (results were likely invalid).

Table 1.2. Sex ratio, fecundity, percent adult mortality, and spore count data for *H. convergens* and three non-target coccinellids infected with a microsporidium under laboratory conditions

	sex ratio (♀:♂)	Fecundity		Mortality (%)		Spore counts (100 µm ²)
		n	Mean ± SE	n	Mortality (%)	
<i>Hippodamia convergens</i>						
control	(22:25)	22	1458 ± 86	22	50.0	
treatment	(21:16)	21	1199 ± 104	21	81.0	101.5 ± 5.6
<i>Adalia bipunctata</i>						
control	(21:21)	21	1327 ± 105	21	47.6	
treatment	(15:17)	15	1385 ± 95	15	60.0	134.4 ± 8.8 ^a
<i>Coccinella septempunctata</i>						
control	(18:16)	17	2111 ± 319	18	38.9	
treatment	(17:15)	16	1900 ± 270	17	35.3	32.4 ± 3.1 ^b
<i>Harmonia axyridis</i>						
control	(22:21)	22	2139 ± 160	22	9.1	
treatment	(20:16)	20	2381 ± 104	20	5.0	24.8 ± 3.3 ^b

¹Two cells (both control and treatment) had expected values less than five (results were likely invalid). ANOVA and Dunnett's multiple comparison with mean spore count of *H. convergens* as a reference: ^a no significant difference, ^b significant difference (P < 0.0001).

Table 1.3. Mean dimensions ($\mu\text{m} \pm \text{SE}$) and measurement ratios of eugregarine trophozoites found in *Addalia bipunctata*.

<i>Gregarine</i>	LP	LD	WP	WD	TL	Range (TL x WD)	TLA	LP:TL	WP:WD
A (n = 22)	12.7 \pm 0.6	42.3 \pm 1.6	15.8 \pm 0.7	26.3 \pm 1.6	55.0 \pm 1.9	34.1-64.9 x 16.5-46.0	4.3	4.3	1.7
B (n = 34)	9.2 \pm 0.3	25.2 \pm 0.7	9.5 \pm 0.3	14.8 \pm 0.6	34.4 \pm 0.9	24.4-44.9 x 9.3-24.5	3.7	3.7	1.6
C Primite (n = 13)	14.3 \pm 1.4	86.3 \pm 6.6	29.2 \pm 3.3	51.2 \pm 5.6	100.7 \pm 7.3	62.2-137.2 x 20.9-77.6	232.5	7.0	1.8
Satellite (n = 2)	18.0 \pm 8.6	113.7 \pm 2.1	46.0 \pm 7.8	70.1 \pm 0.3	131.8 \pm 10.6	121.2-142.4 x 69.8-70.5	7.3	7.3	1.5

LP, protomerite length; LD, deuteromerite length; WP, primite width; WD, deuteromerite width; TL, total trophozoite length; TLA, total length of association.

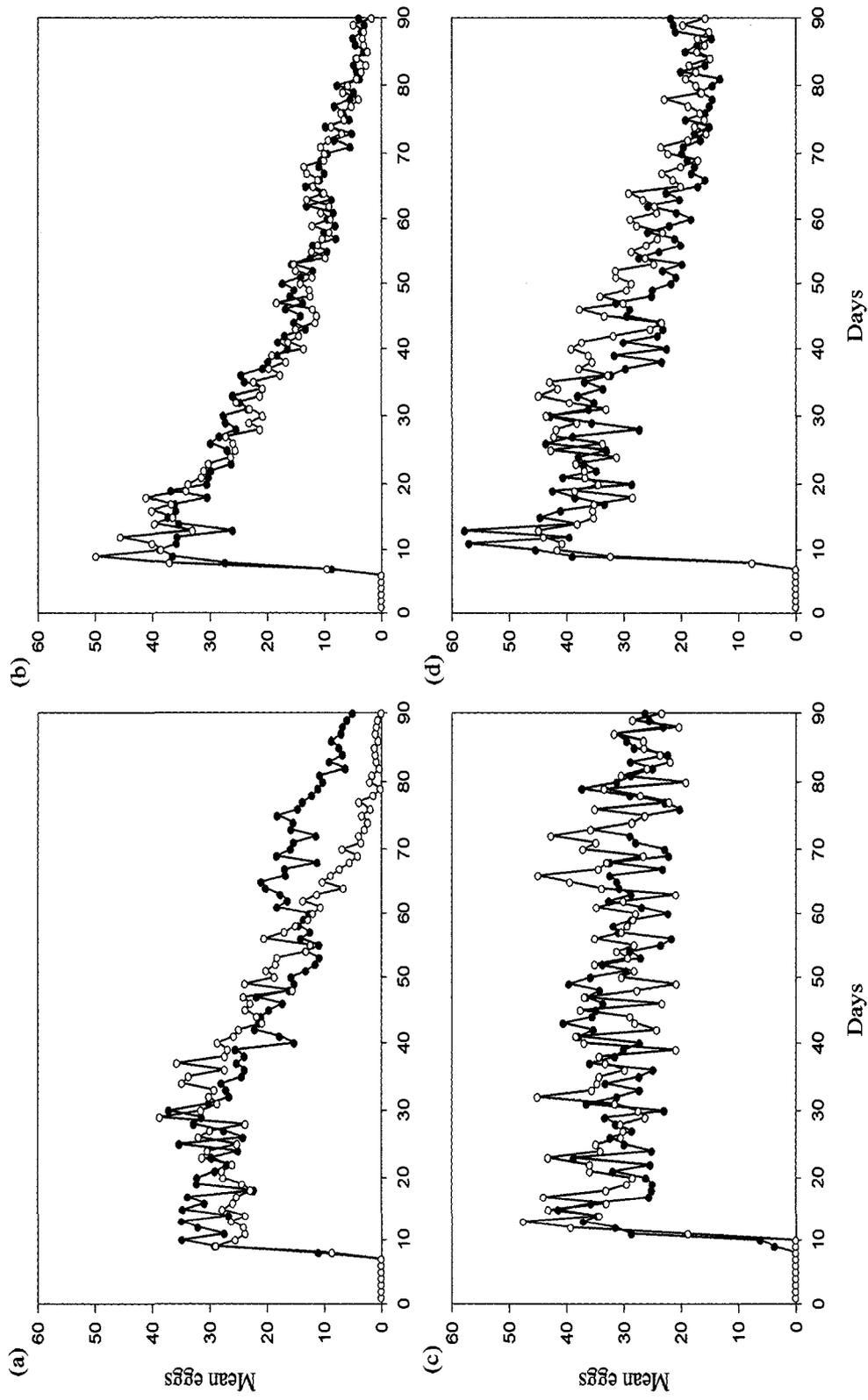


Fig. 1.1. Age-specific oviposition curves (mean eggs produced per day) of uninfected and microsporidia-infected females during a 90-day trial. Solid circles, uninfected; clear circles, microsporidia-infected. (a) *H. convergens* ($n = 22$ uninfected, 21 infected), (b) *A. bipunctata* ($n = 17, 16$), (c) *C. septempunctata* ($n = 17, 16$), (d) *H. axyridis* ($n = 22, 20$).

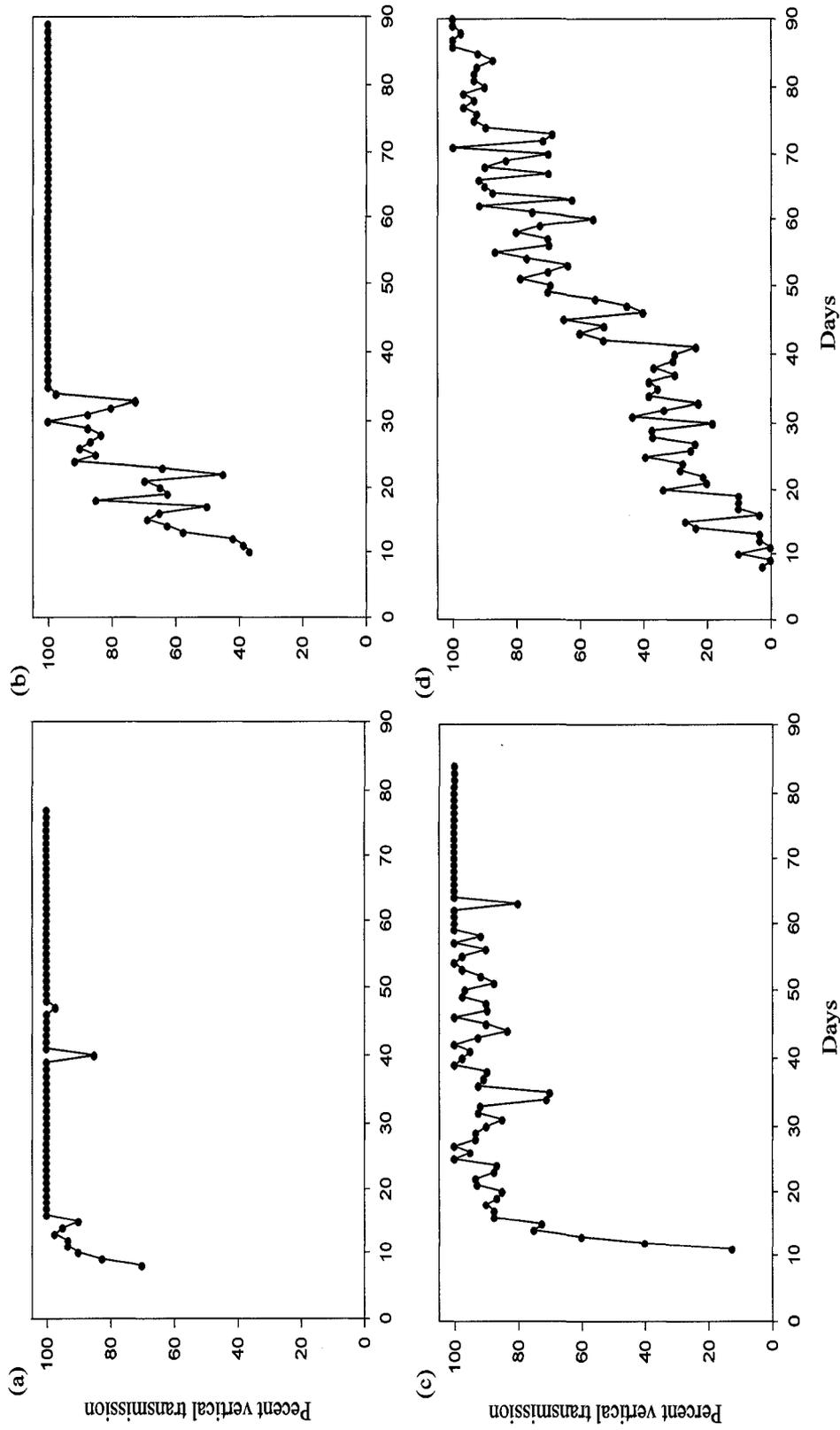


Fig. 1.2. Vertical transmission of microsporidia (percent microsporidia-infected eggs) by coccinellids that were infected with microsporidia under laboratory conditions (a) *H. convergens*, (b) *A. bipunctata*, (c) *C. septempunctata*, (d) *H. axyridis*. Beetles were mated six days following eclosion (Day 0).

Chapter 2:

Tissue pathology of a microsporidium in the convergent lady beetle,
Hippodamia convergens Guérin-Ménéville (Coleoptera: Coccinellidae)
and in three non-target coccinellids

2.1. Introduction

Three species of microsporidia have been described from coccinellids. *Nosema hippodamiae* Lipa and Steinhaus was described from the midgut, fat body and other unspecified tissues of *H. convergens* (Lipa and Steinhaus, 1959). *N. tracheophila* Cali and Briggs infects the tracheal epithelium, hemocytes and connective tissues of *Coccinella septempunctata* L. (Cali and Briggs, 1967) and *N. coccinellae* Lipa is known to infect the midgut epithelium, Malpighian tubules, gonads, nerves and muscle of *C. septempunctata*, *Hippodamia tredecimpunctata* L. and *Myrrha octodecimguttata* L. (Lipa, 1968a). *N. coccinellae* was later reported from *C. quinquepunctata* L., *Adalia bipunctata* L., and *Exochomus quadripustulatus* L. (Lipa *et al.*, 1975).

The unidentified microsporidium from *H. convergens* has a relatively broad host range (Saito and Bjørnson, 2006; previous chapter). The pathogen was transmitted horizontally to several non-target hosts (*A. bipunctata*, *C. septempunctata*, *C. trifasciata perplexa*, and *H. axyridis*) under laboratory conditions. Once infected, the microsporidium is transmitted vertically, allowing the pathogen to persist in non-target host populations.

Many species of microsporidia have been described by examining their life cycle and tissue pathology by light microscopy, and pathogen ultrastructure by transmission

electron microscopy. In recent studies, molecular characterization of the pathogen is deemed as an important method for species differentiation of these pathogens (Franzen and Müller, 1999). However, past descriptions of microsporidia from coccinellids were based solely on pathogen life cycle, spore dimensions and a description of the tissues that are infected. These past studies were undertaken during the 1950s and 60s when transmission electron microscopy was not widely used and molecular techniques had not yet been developed. Therefore, an examination of tissue pathology by light microscopy is essential to help distinguish the microsporidium from *H. convergens* in this study from those that were previously described from *H. convergens* and other coccinellids. Therefore, the objective of this study is to determine which tissues of *H. convergens* are invaded by the microsporidium and to examine the tissues that are infected in susceptible non-target coccinellids (*A. bipunctata*, *C. septempunctata*, and *H. axyridis*).

2.2. Materials and Methods

Beetles were reared and maintained as described in the previous chapter. Ten *H. convergens* virgin females were randomly selected from uninfected and microsporidia-infected laboratory colonies. Females were mated with males from the uninfected colony and mated pairs were provided aphids, artificial diet (Lacewing & Ladybug Food, honey, and distilled water) and distilled water for a minimum of seven days. Females were confirmed as either infected or not by examining their eggs for microsporidian spores. Uninfected *H. convergens* females (controls) were examined to detect any tissue abnormalities in microsporidia-infected conspecifics, and infected *H. convergens* were used for making comparisons with infected non-target species.

For all of the non-target species examined, uninfected females were obtained from laboratory colonies. Microsporidia-infected females were obtained through the following process: (1) uninfected larvae were fed two microsporidia-infected *H. convergens* eggs ($n = 40$) and reared to adults; (2) 10 females were randomly selected and mated (they were confirmed to be infected by examining their eggs for microsporidian spores); and (3) their offspring were reared to adults. Ten infected females (resulting from vertical transmission) were obtained and confirmed to be infected by examining their eggs for microsporidian spores.

For each species, 20 specimens (10 uninfected and 10 infected) were fixed with Carnoy's fixative and embedded in Paraplast® Plus (Sigma-Aldrich Inc., melting point 56 °C) but only eight specimens (4 uninfected, 4 infected) were sectioned with a rotary microtome (5 μm). Prior to processing, the head, pronotum and legs of each specimen were removed. Excision of these body parts helped to ensure maximum infiltration of the fixative. Whole abdomens of female beetles were embedded and examined. Four specimens were sectioned longitudinally (2 uninfected, 2 infected) and the other four were sectioned parasagittally (see Appendix A). Eight slides were prepared for each specimen and 12 sections (two rows of six consecutive sections) were mounted on each slide. Sections were stained with Harris hematoxylin (Fisher Scientific, SH30-500D) and Alcoholic Eosin Y (Fisher Scientific, SE22-500D) and examined by light microscopy (see Appendix B).

2.3. Results

Organs and tissues in *H. convergens* were identified according to Landis (1936), Pradhan (1939), and Rothschild *et al.* (1986) (Fig. 2.1 and 2.2). Microsporidian spores were observed in longitudinal muscle surrounding the midgut (Fig. 2.3a) and within the fat body (Fig. 2.3b), Malpighian tubules (Fig. 2.3c), pyloric valve epithelium (Fig. 2.3d), (Fig. 2.4a), ventral nerve cord ganglia (Fig. 2.4b), muscles (Fig. 2.4c), (Fig. 2.5a), and ovaries (Fig. 2.5b) (Table 2.1). With exception of the colon epithelium and connective tissues, microsporidian spores were observed in all of the tissues sections that were examined. In one four specimens of infected *H. convergens* females, infected cells were not observed in the colon epithelium. Connective tissues were rarely invaded . Significant tissue abnormalities were not observed when uninfected tissues (from *H. convergens* controls) were compared to the same tissues in microsporidia-infected *H. convergens* females. However, infected host cells of susceptible organs were occasionally hypertrophic due to the large number of spores filling the cells (Fig. 2.3d and 2.4a). Basic insect immune responses, such as encapsulation and melanization, were not observed.

The orientation and appearance of the organs and tissues in the non-target hosts (*A. bipunctata*, *C. septempunctata* and *H. axyridis*) were similar to those of the natural host but tissue pathology among some of the non-target hosts differed (Table 2.1). The same tissues were infected in *A. bipunctata* as in the natural host, *H. convergens*. The pyloric valve epithelium and colon epithelium were not infected in one microsporidia-infected *A. bipunctata* female that was examined. Connective tissues were rarely invaded. In the case of *C. septempunctata*, microsporidian spores were not observed at all in the

colon epithelium. The pyloric valve epithelium remained uninfected in one specimen. Infection in *H. axyridis* was least similar to the natural host. Spores were not observed in the colon epithelium or connective tissues of any of the specimens, and spores were observed less frequently in the Malpighian tubules of *H. axyridis* than in other non-target hosts. For all species examined, only focal infections were observed (portions of susceptible organs were infected). The major sites of infection, based on the observed spore load and frequency, were: the fat body, Malpighian tubules, pyloric valve epithelium, muscles and ovaries.

2.4. Discussion

Tissue pathology is the only clear distinction among the three microsporidia (*Nosema hippodamiae*, *N. coccinellae*, and *N. tracheophila*) that have been described from coccinellids (Lipa and Steinhaus, 1959; Cali and Briggs, 1967; Lipa, 1968a) (Table 2.1). Although these microsporidia infect different host species, host ranges were not investigated and it is possible that each microsporidium invades specific host tissues in different hosts. For example, Lipa (1968) found *N. coccinellae* in *C. septempunctata* and *Myrrha octodecimguttata* but the pathogen infected different tissues in each host. An examination of tissue pathology among the four coccinellids during this study provides further evidence that one microsporidium may infect distinct tissues in different hosts.

An examination of tissue pathology in *H. convergens* did not provide conclusive evidence regarding the identity of the microsporidium. *N. hippodamiae* (host: *H. convergens*) infects the midgut epithelium, fat body, and other unspecified tissues (Lipa and Steinhaus, 1959) (Table 2.1). The microsporidium examined in this study did not

infect the midgut epithelium but spores were present in the muscles surrounding the midgut (Fig. 2.3a) and were also observed within the fat body (Fig. 2.3b), Malpighian tubules (Fig. 2.3c), pyloric valve epithelium (Fig. 2.3d), colon epithelium (Fig. 2.4a), ventral nerve cord ganglia (Fig. 2.4b), muscles (Fig. 2.4c), connective tissues (Fig. 2.5a), and ovaries (Fig. 2.5b).

N. tracheophila spores are found in the tracheal epithelium, haemocytes and connective tissues of *C. septempunctata* but this pathogen does not infect the fat body (Cali and Briggs, 1967) (Table 2.1). The unidentified microsporidium in the current study was not observed in the tracheal epithelium but spores were observed in the connective tissues of *C. septempunctata*. Hemocytes were not observed in the tissue sections in this study; however, microsporidian spores were observed in abundance throughout the fat body.

N. coccinellae is reported to infect the midgut epithelium, Malpighian tubules, gonads, nerves and muscle tissues of several coccinellids, including *A. bipunctata* and *C. septempunctata* (Lipa, 1968a) (Table 2.1) but the fat body is not invaded by *N. coccinellae*. Tissue pathology of the unidentified microsporidium in *A. bipunctata* and *C. septempunctata* was similar. The pathogen invaded the fat body; however, the midgut epithelium was not infected. Microsporidia-infected specimens examined during this study were obtained through vertical transmission of the pathogen from infected females, which means that the pathogen was present during embryogenesis. These specimens did not become infected directly from ingesting microsporidia-infected food (horizontal transmission). This may explain, in part, why spores were not observed in the midgut

epithelium. Nevertheless, there were opportunities for infected females to cannibalize their own infected eggs while they were being maintained individually in cups.

The midgut epithelium is the primary site of enzymatic digestion and the absorption of nutrients. The midgut epithelium is not lined with cuticle and this may make it vulnerable to infection following the ingestion of viable spores. Despite this perceived vulnerability, microsporidian spores were not observed in the midgut epithelium of *H. convergens* or the three non-target hosts that were examined. For all species examined, the midgut epithelium and food bolus were separated from the midgut epithelium by a peritrophic membrane. This nonliving, permeable network of chitin and protein is secreted by the midgut epithelium. It functions to reduce abrasion of the midgut epithelium and inhibits pathogen entry to the epithelial cells (Triplehorn and Johnson, 2005). The peritrophic membrane may have prevented pathogen invasion of the midgut epithelium.

Organs that were infected by the microsporidium are not involved in nutrient absorption. Rather, the pathogen invades organs that act as a food reservoir and are responsible for intermediate metabolism (fat body), excretion (Malpighian tubules and hindgut), movement (nerve and muscle), sense (nerves), and reproduction (ovary). The tissues that are invaded by the pathogen may inadvertently maximize the reproductive success of the pathogen. By not interfering with the efficiency of the host to acquire energy, the pathogen has a constant energy supply for continual reproduction as it remains sub-lethal to the host. The host is able to survive and produce offspring and vertical transmission of the pathogen is facilitated because the ovary and eggs are invaded.

The infection of the nerve (Fig. 2.4b) may alter the behaviour of microsporidia-infected females. Although the observation was not quantified, infected females were often observed mounting other females in an attempt to mate. Such behaviour is unusual for females; however, it may ultimately increase pathogen transmission because this mounting behaviour involves close contact of the infected female to other females. Spores of the microsporidium were detected in coxal fluid and fecal matter of infected *H. convergens* (Joudrey, 2006). The body surface of infected female beetles may be covered with infective spores; therefore female mounting behaviour may facilitate horizontal transmission of the pathogen. In addition, mobility may be adversely affected because microsporidian spores were observed within the flight/walking muscles (Fig. 2.4c). Masses of spores, were observed throughout the muscle tissue (Fig. 2.4c) and the pressure from these spores may hinder muscle performance.

There are two routes of vertical transmission. Transovum transmission occurs when the pathogen adheres to the egg surface, whereas transovarial transmission occurs when the pathogen resides within the ovary and eggs (Andreadis, 1987). Microsporidian spores were clearly observed within the ovaries and eggs (Fig. 2.5b); therefore, the microsporidium was transmitted transovarially in all the beetle species examined.

The observed differences of tissue pathology among the natural and non-target hosts do not seem significant enough to provide an explanation of why the longevity and fecundity of only the natural host (*H. convergens*) was affected by the microsporidium (see previous chapter). *A. bipunctata* (a native species) showed the same tissue pathology with the natural host. Spore counts and the duration for the pathogen to reach 100% vertical transmission were also similar (previous chapter). It would be interesting

to determine why *A. bipunctata* seems to be unaffected by the microsporidium in terms of longevity and fecundity. Conversely, the pathogen infected fewer tissues in *C. septempunctata* and *H. axyridis* (introduced species) than in *A. bipunctata* and *H. convergens*. This appears to reflect the relative unsuitability of these introduced beetle species for the microsporidium (previous chapter, low spore counts and longer times for the pathogen to reach 100% vertical transmission). Although *C. septempunctata* and *H. axyridis* are less susceptible to the microsporidium, they are able to transmit the pathogen transovarially.

The host range for the unidentified microsporidium in *H. convergens* overlaps with the host ranges reported for the three previously-described species (*N. hippodamiae*, *N. tracheophila*, and *N. coccinellae*) (Table 2.1). Based on the outcome of this study, an examination of tissue pathology does not provide the evidence required to make conclusions regarding the identity of the undescribed microsporidium in *H. convergens*. Pathogen ultrastructure and molecular characterization is required for this pathogen to be described formally and for comparisons to be made with type specimens of the three previously-described microsporidia from coccinellids.

Table 2.1. Host range, spore dimensions and tissue pathology of microsporidia from coccinellid hosts

Microsporidia & Their Host Ranges	Spore Size (fixed and stained, µm)	Tissues Infected
<i>Nosema hippodamiae</i> ¹ <i>Hippodamia convergens</i>	3.3-5.4 x 2.2-2.7	midgut, fat body
<i>Nosema tracheophila</i> ² <i>Coccinella septempunctata</i>	3.7 x 2.3	tracheal epithelium, blood cells, connective tissues
<i>Nosema coccinellae</i> ³ <i>Adalia bipunctata</i> L. ⁴ <i>C. quinquepunctata</i> L. ⁴ <i>C. septempunctata</i> <i>Exochomus quadripustulatus</i> L. ⁴ <i>H. tredecimpunctata</i> L. <i>Myrrha octodecimguttata</i> L.	3.6-6.2 x 2.0-3.6	midgut epithelium, Malpighian tubules, gonads, nerves, muscle tissues
Unidentified microsporidium <i>H. convergens</i> , <i>A. bipunctata</i> ⁶ , <i>C. septempunctata</i> ⁶ <i>C. trifasciata perplexa</i> ^{6,7} , <i>Harmonia axyridis</i> ⁶	3.9 x 2.5 ⁵	pyloric valve epithelium, colon epithelium ⁸ , Malpighian tubules, ovaries, fat bodies, connective tissues ⁹ , muscles, nerves

¹ Lipa and Steinhaus, 1959; ² Cali and Briggs, 1967; ³ Lipa, 1968; ⁴ Lipa *et al.*, 1975; ⁵ Joudrey (2006); ⁶ infection resulting from the consumption of microsporidia-infected *H. convergens* eggs; ⁷ not subjected to a histological study; ⁸ not observed in *C. septempunctata* and *H. axyridis*; ⁹ not observed in *H. axyridis*.

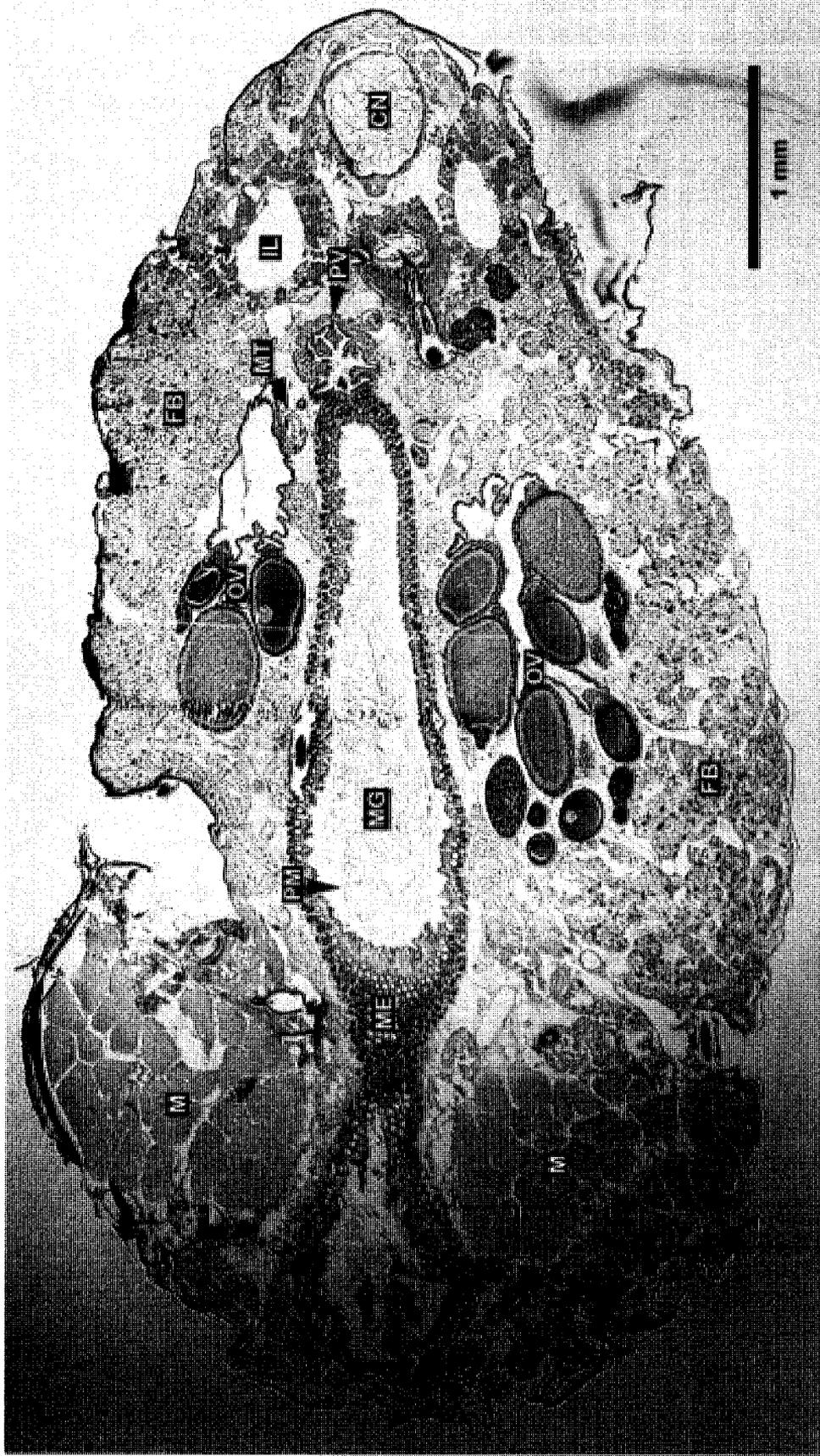


Figure 2.1. Longitudinal section of microsporidia-infected *H. convergens*. CN, colon; FB, fat body; IL, ileum; M, muscle; ME, midgut epithelium; MG, midgut; MT, Malpighian tubule; OV, ovary; PM, peritrophic membrane; PV, pyloric valve.



Figure 2.2. Parasagittal section of microsporidia-infected *H. convergens*. CN, colon; IL, ileum; M, muscle; ME, midgut epithelium; MG, midgut; MT, Malpighian tubule; NC, ventral nerve cord; OV, ovary; PV, pyloric valve.

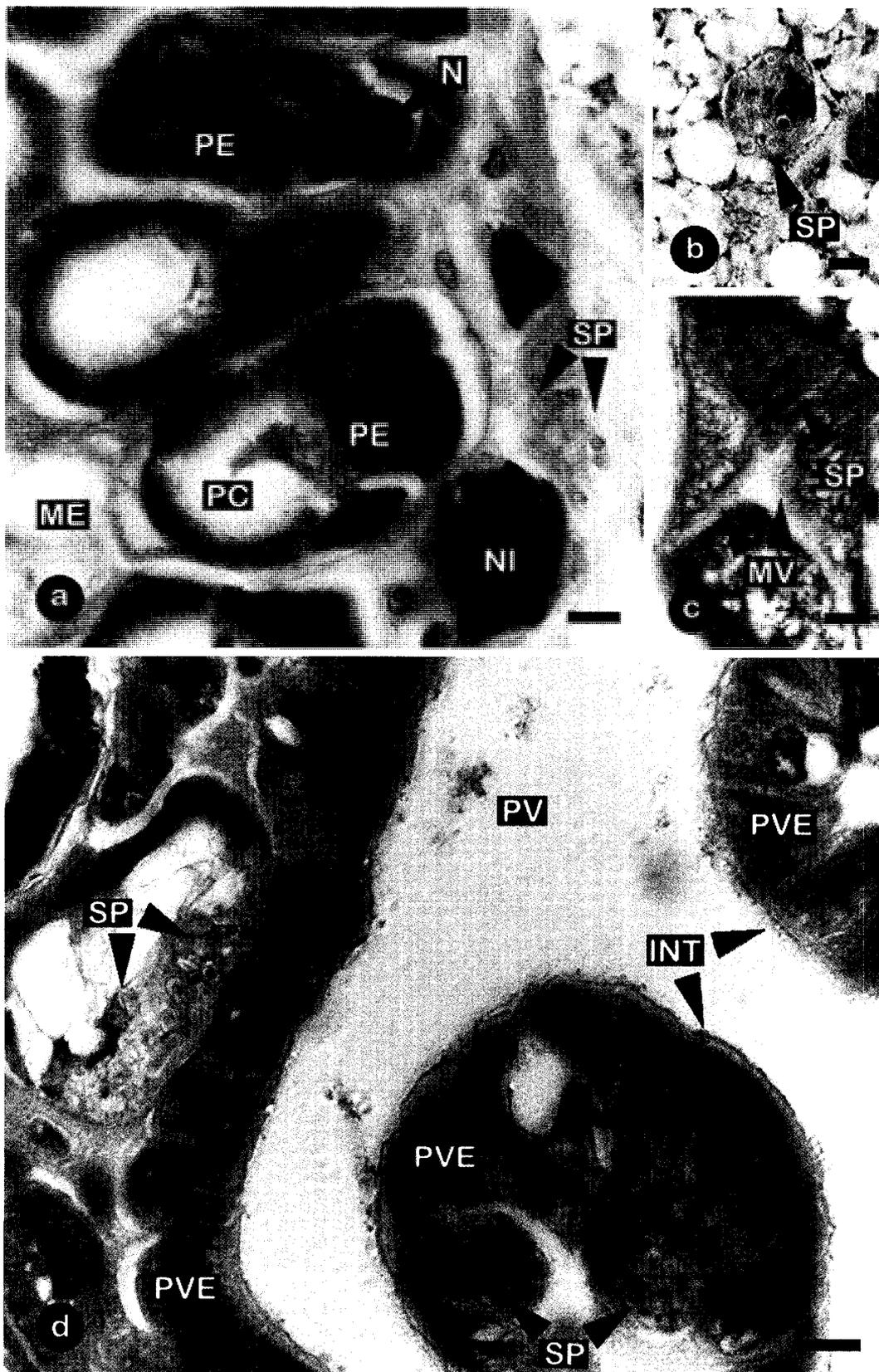


Figure 2.3. Microsporidia-infected tissues in *H. convergens*. Spores within the (a) longitudinal muscle surrounding the midgut; (b) fat body; (c) Malpighian tubule (cross section); and (d) pyloric valve epithelium. INT, intima (cuticular lining); ME, midgut epithelium; MV, microvilli; N, nucleus; NI, nidi; PC, pro-epithelial cavity; PE, pro-epithelial cells; PV, pyloric valve; PVE, pyloric valve epithelium, SP, microsporidian spores. Scale bars, 10 μ m.

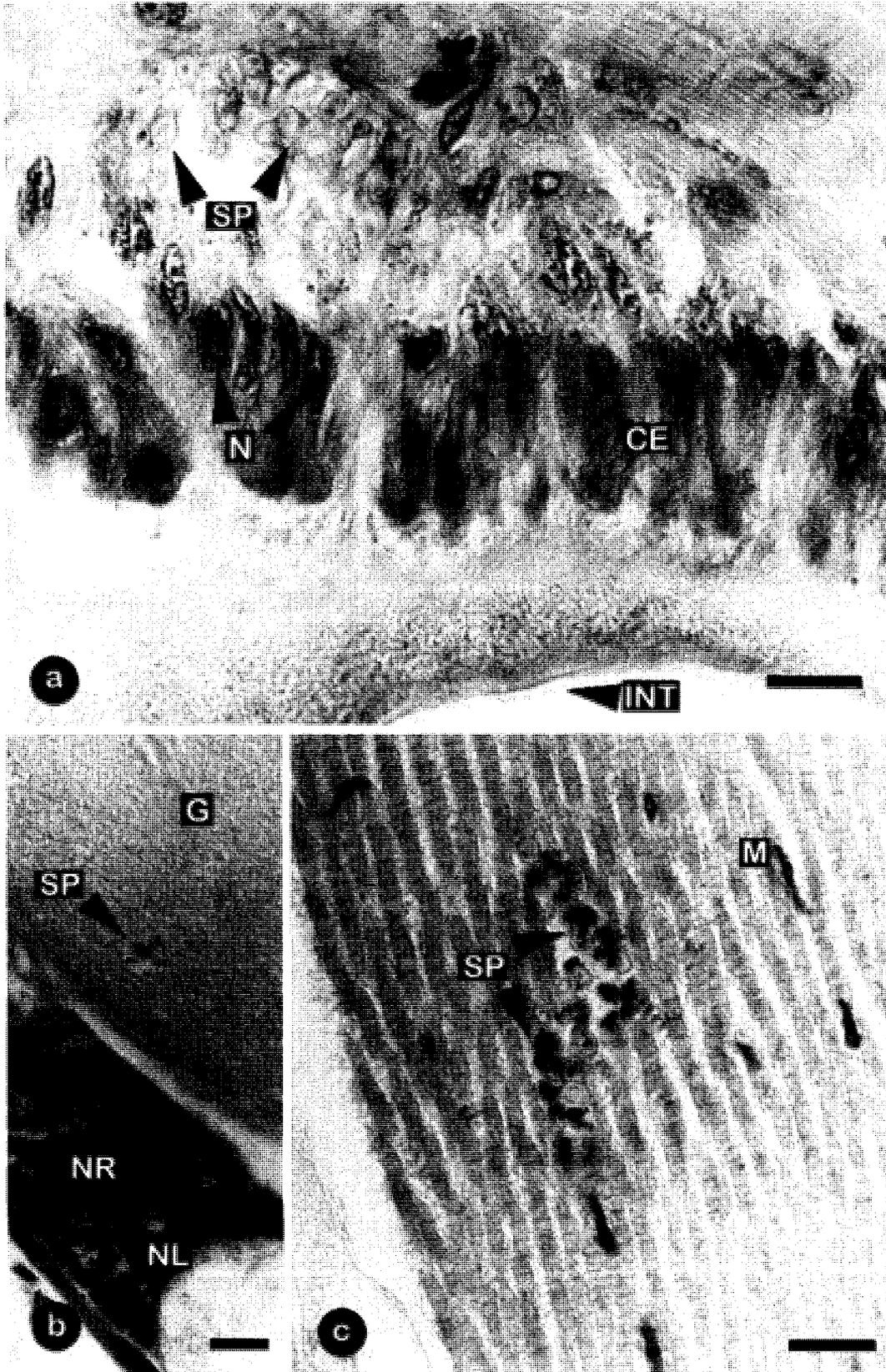


Figure 2.4. Microsporidia-infected tissues in *H. convergens*. Spores within the (a) colon epithelium; (b) ventral nerve cord ganglion; and (c) muscle. CE, colon epithelium; G, ganglion; INT, intima; M, muscle; N, nucleus; NR, neuron; NL, neural lamella; SP, microsporidian spores. Scale bars, 10 μm.

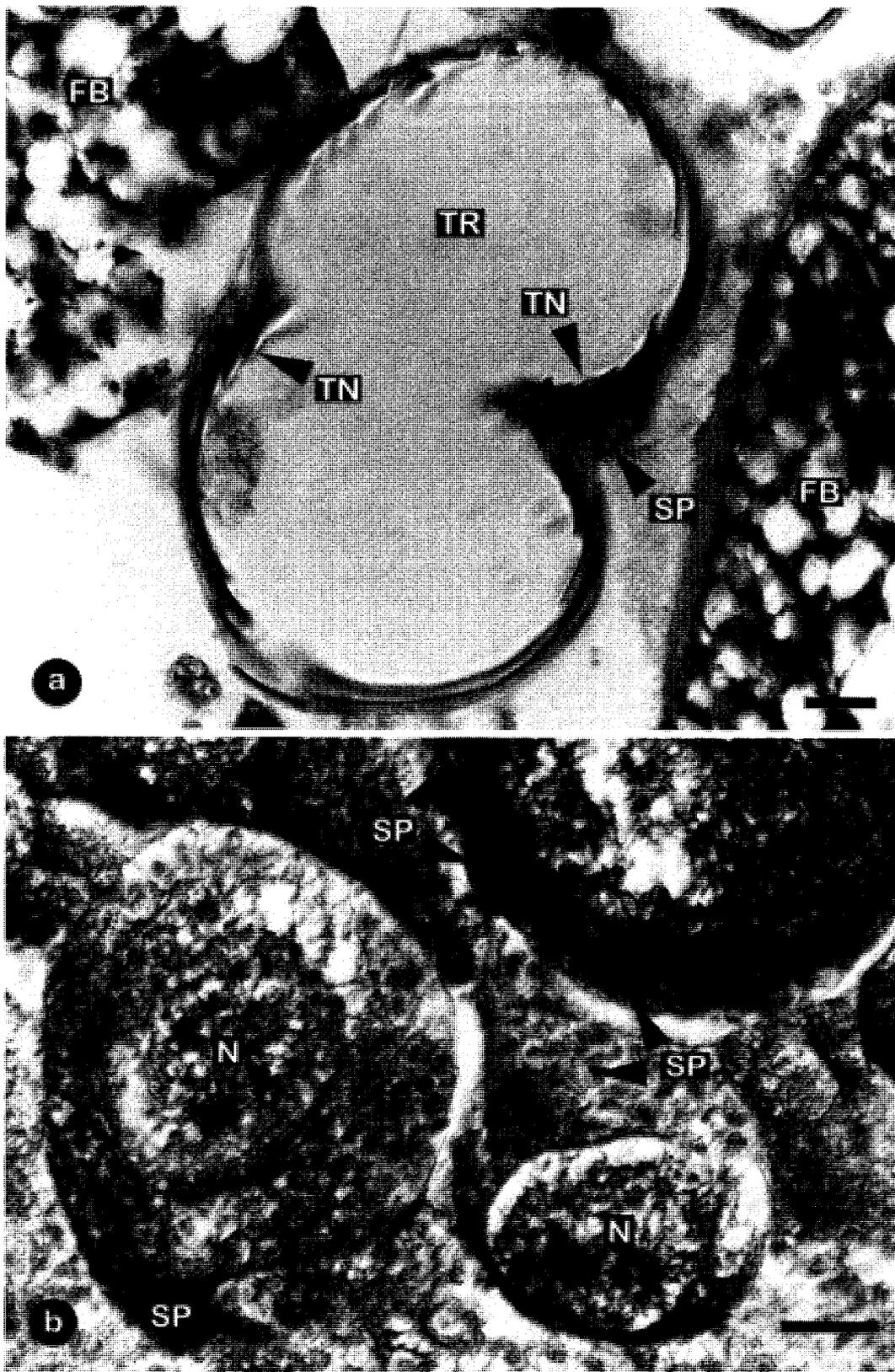


Figure 2.5. Microsporidia-infected tissues in *H. convergens*. Spores within the (a) connective tissue around a trachea; and (b) developing oocyte (immature egg). FB, fat body; N, nucleus; SP, microsporidian spores; TN, taenia (spiral thickening of the trachea), TR, trachea. Scale bars, 10 μ m.

Conclusion

The microsporidium from *H. convergens* prolonged larval development in *H. convergens* and in the three non-target hosts examined during this study (*A. bipunctata*, *C. septempunctata* and *H. axyridis*) (Table 1.1). The pathogen reduced the fecundity and adult longevity of *H. convergens*, an observation that is consistent with the outcome of a previous study (Joudrey and Bjørnson, 2007). However, the pathogen had no apparent effect on the fecundity or longevity of the three non-target host species (Table 1.2).

Microsporidia lack mitochondria and are dependent on their hosts for the energy that is required for their development. In most cases, microsporidia cause sub-lethal effects without killing their hosts. The microsporidium found in *H. convergens* is able to develop within the three non-target hosts that were studied but the energy used by the pathogen does not appear to affect the ability of these beetles to reproduce or survive. However, the microsporidium may affect other life history characteristics, such as mobility or prey consumption that were not quantified during this study. Furthermore, the microsporidium may have more pronounced effects on the non-target beetles if they are subjected to a stressful rearing environment. Beetles in this study were fed an abundance of food and were reared under optimal conditions.

Vertical transmission of the microsporidium was observed for all of the host species that were examined. The duration for vertical transmission to reach 100% depended on the host that was infected (Fig. 1.2). Vertical transmission patterns seemed to reflect the susceptibility of a particular beetle species to the microsporidium. Results showed that the introduced and highly invasive coccinellid species (*C. septempunctata* and *H. axyridis*) were less suitable as hosts for this microsporidium than was the native

species (*A. bipunctata*) or the natural host (*H. convergens*). The aggressiveness of these introduced beetles (in combination with their ability to produce more eggs than native species and their ability to withstand the effects caused by the microsporidium), may favor them over native species with respect to resource competition and survival.

An examination of tissue pathology did not provide the information that was needed to distinguish the unidentified pathogen from *H. convergens* from those that were previously described from *H. convergens* and other coccinellids. The pathogen from *H. convergens* infected fewer tissues in *C. septempunctata* and *H. axyridis* than in *A. bipunctata*. This suggests that the introduced beetle species were less suitable hosts for the microsporidium than was the native species.

Host range, spore dimensions and tissue pathology of this microsporidium overlap, in part, with those of the three previously described microsporidian species (Table 2.1). The geographical locality of these pathogens is distinct: *Nosema hippodamiae* and *N. tracheophila* are reported from the USA, whereas *N. coccinellae* is reported from Europe. However, *N. coccinellae* may have been transported inadvertently from Europe to North America because *A. bipunctata* (a known host of *N. coccinellae*) is commercially available from Europe for aphid control in North America. It is possible that the unidentified microsporidium in *H. convergens* is the same as the other three species of microsporidia reported from coccinellids. It is also possible that the unidentified microsporidium represents a new species.

Microsporidian spores were not observed in the midgut epithelium. For all host species examined, the midgut epithelium and food bolus were separated from the midgut epithelium by a peritrophic membrane, which is secreted by the midgut epithelium. The

peritrophic membrane helps to reduce abrasion of the midgut epithelium and inhibits pathogen invasion of the midgut epithelium (Triplehorn and Johnson, 2005). The pathogen invaded organs that are not involved in nutrient absorption, including the fat body (food reservoir and intermediate metabolism), Malpighian tubules (excretion), hindgut (water resorption and excretion), nerve (sense and mobility), muscle (mobility), and reproduction (ovary). By invading these tissues, the microsporidium has the energy required for continual reproduction as it remains sub-lethal to the host. The host is able to survive and produce infected offspring, facilitating vertical transmission of the pathogen.

The consumption of only one microsporidia-infected *H. convergens* egg by a susceptible, non-target coccinellid may result in infection of that host (horizontal transmission). Once these new hosts are infected, the pathogen is capable of spreading throughout the beetle population when it is transmitted vertically in subsequent generations. This scenario is likely to occur under natural conditions because there is a tendency among coccinellids for cannibalism and intra guild predation (Agarwala, 1991, 1998; Agarwala and Dixon, 1992; Gupta *et al.*, 2006).

The results of this study raise concerns regarding the current method of collecting and importing *H. convergens* for biological pest control. Collecting beetles from their overwintering sites for distribution without strict quarantine protocols may result in the inadvertent importation and release of microsporidia (Bjørnson, 2008). Field-collected coccinellids that are used for biological control must be examined for microsporidia and other pathogens prior to their release. Uninfected individuals should be used to establish

rearing colonies for the production of disease-free beetles for release in biological pest control programs.

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Appendix A

Embedment and sectioning of tissues in paraffin (modified from Becnel, 1997)

1. Living insect hosts were fixed in Carnoy's fixative (60 ml absolute ethanol, 30 ml chloroform, 10 ml glacial acetic acid). While in the fixative, the head and pronotum of each specime was removed, followed by the elytra and hind-wings. The legs were removed from the base of each coxa to ensure that the resulting openings were exposed to the fixative. Additional openings were made by inserting a fine dissecting needle under metasternum and making small incisions on the both side of abdomen between ventrite 4 and 5.

2. Specimens were soaked in 70% ethanol overnight. At this point, the tissue may be stored in 70% ethanol at room temperature for extended period of time.

3. Specimens were dehydrated to tertiary-butyl alcohol and infiltrated with Paraplast® Plus (Sigma-Aldrich Inc., melting point 56 °C):

80% ethanol	2 h
95% ethanol	2 h
100% ethanol	1 h (twice)
100% ethanol: 100% butanol (1:1)	Overnight
100% butanol (above 25.5 °C)	2 h (twice)
100% butanol in 60 °C oven	2 h
100% butanol: Paraplast (3:1) in 60 °C oven	20 min
100% butanol: Paraplast (1:1) in 60 °C oven	Overnight
Paraplast in 60 °C oven	2 h
Paraplast in 60 °C oven under vacuum	2 h

4. Embedding containers were made by folding paper into a rectangular container with tabs on both ends. Specimens were embedded in fresh Paraplast, with the sample near (but not on) the bottom of the container. This was done by pouring a small amount of the Paraplast into the container and allowing it to harden slightly before adding the sample and the remainder of the paraffin. Once the tissue was added, it was important for the Paraplast that contains the specimen to be cooled rapidly. Paper containers were held on the surface of a cold water bath until a uniform film was formed on the surface of the paraffin. The containers were then submerged in the cold water and allowed to harden.

5. After the paraffin hardened, the container was removed and a block was trimmed to expose the tissue. A rotary microtome (820 Spencer Microtome, American Optical Corporation) was used to make sections. A sheet of paper was placed by the microtome to catch the sections. In order to obtain ribbons of sections and avoid curling, a fine brush was used to gently lift the ribbon while new sections were continuously produced.

6. Microscope slides were coated with thin film of protein solution (1g gelatin, 2g solid phenol, 15 ml glycerin, 100 ml distilled water) so that the sections would adhere to the slides. Before mounting the sections, a few drops of 1% formalin were placed on the slide so that the sections would float on the slide surface.

7. Sections were placed on the microscope slides by carefully picking the ribbons up with a scalpel. The sections were then warmed on a slide warmer (50 °C, Fisher Scientific, Model 77) to flatten, straighten and fix the ribbons to the slide. Sections were left on the slide warmer overnight to dry completely (40 °C).

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Appendix B

Rehydration and staining of slides using hematoxylin and eosin stains

1. Before staining, the paraffin was removed from the sections with xylene. The sections were then rehydrated through a decreasing ethanol concentration series to distilled water as described below:

100% xylene	2 h (twice)
100% xylene: 100% ethanol (1:1)	3 min
100% ethanol	3 min
95% ethanol	3 min
70% ethanol	3 min
50% ethanol	3 min
Distilled water	3 min

2. Sections were stained with Hematoxylin and Eosin as described below:

Harris hematoxylin solution	
(Fisher Scientific, SH30-500D)	6 min
Rinse in running tap water	30 sec
Differentiating solution	
(0.25 ml HCl, 100 ml 70% ethanol)	3 dips
Rinse in running tap water	30 sec
Blue in Scott's tap water substitute solution	
(500 ml tap water, 5 g magnesium sulphate, 1 g sodium bicarbonate)	30 sec
95 % ethanol	30 sec
Alcoholic Eosin Y	
(Fisher Scientific, SE22-500D)	30 sec
95% ethanol	1 min (twice)
100% ethanol	1 min (twice)
100% xylene	10 min