Intraspecies Transmission and Effects of an Unidentified Microsporidium on the Convergent Lady Beetle, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), Used for Biological Control

By

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Intraspecies Transmission and Effects of an Unidentified Microsporidian on the Convergent Lady Beetle, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), Used for Biological Control

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Convergent lady beetles, *Hippodamia convergens* Guérin-Méneville, are often used for aphid control in agriculture and in home gardens. Beetles are collected from overwintering sites in California and are customarily released in large quantities. Although *H. convergens* are known to host the microsporidium *Nosema hippodamiae*, there are currently no screening procedures in place to ensure beetle health or quality. The purpose of this study was to examine the effects of an unidentified microsporidium found in commercially-available *H. convergens* on its life history characteristics and to examine vertical and intraspecies horizontal transmission routes. Microsporidia-infected *H. convergens* larvae took significantly longer to develop than did their uninfected cohorts. There was no significant difference in mean egg hatch and larval mortality. Cumulative mean egg production and mean adult survival for microsporidia-infected females was significantly lower than that of uninfected females. Vertical transmission was 100% efficient and horizontal transmission occurred through ingestion of spores. Microsporidian spores in this study measured 3.6 x 2.4 μm, which is within the range reported for *N. hippodamiae* measure.

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Introduction

Over the last few decades, a common solution for many insect pest problems was to treat the infested area with insecticides. While this practice may have been effective some of the time, it often resulted in an increase in pest numbers or allowed new pests to become established (DeBach and Rosen, 1991). Many insecticides are not host specific and kill a variety of insects in the area where they are applied, including the beneficial insects that maintain a pest species at their economic threshold (the point where the pest species does not cause enough damage to induce economic loss) (DeBach and Rosen, 1991; Dixon, 2000).

Even when an insecticide affects a single target species, its use may result in the dispersal of the natural enemies that normally keep the pest population under control. In this situation, the natural enemies of the pest no longer have a food source to sustain them (Obrycki and Kring, 1998). Without its natural enemies present, the pest will be able to recover and flourish in greater numbers than before the insecticide was applied (DeBach and Rosen, 1991). Furthermore, insecticides rarely kill every individual of the pest population and individuals that survive often develop some form of resistance to the chemical that was applied (DeBach and Rosen, 1991). The efficacy of future applications of the same insecticide is often reduced because the few pest individuals that remain will eventually repopulate the area with a new generation of insecticide-resistant offspring.

Many cities worldwide, including the Halifax Regional Municipality, have imposed restrictions regarding the use of insecticides due to a public perception that they may cause unforeseeable, long-term environmental effects. With the rules and

regulations governing the use of insecticides becoming stricter, integrated pest management (IPM) has received greater attention as a viable alternative to chemical pest control. IPM is a system approach to pest management that involves the implementation of a variety of control measures to keep pest populations below their economic thresholds. Chemical control is used in moderation only when other control techniques have been deemed ineffective (Dent, 2000). The use of natural enemies for pest control, otherwise known as biological control, is part of the IPM approach to pest management and is considered one of the least invasive forms of pest control (DeBach and Rosen, 1991; Dixon 2000).

There are three different types of biological control: augmentative, classical, and conservative. Augmentative biocontrol refers to the practice of increasing the numbers of native natural enemies to control a pest species within a particular area. Conversely, classical biocontrol refers to the use of imported natural enemies for pest control. This method is especially effective when the target pest is not native to the infested area (Dixon, 2000). Conservative biological control is the least invasive form and does not directly deal with insects. Instead, the focus is to improve farming and gardening techniques by promoting a more suitable environment for natural enemies to inhabit (Mahr and Ridgway, 1993). All three types of biological control rely on three different types of natural enemies for pest control: predators, parasitoids, and pathogens (DeBach and Rosen, 1991).

Predators, as the name implies, actively search for their prey and feed directly on the pest species. Predators consume more than one individual of a prey species each day and will generally attack more than one life stage of their prey (DeBach and Rosen,

1991). Additionally, predatory natural enemies usually feed as both immatures and adults (DeBach and Rosen, 1991; Dixon, 2000). As a result, adult predators do not need to disperse to locate a food source for their immature stages and eggs are often laid near the pest on which the immature stages feed.

Parasitoids spend at least part of their lifecycle living in or on the pest and will only feed on one prey individual during their larval period. Following eclosion, most adult parasitoids do not continue to feed on the same host. Although a parasitoid generally kills only one pest during its lifetime, parasitoids generally have high fecundity and short lifecycles, which allow many generations to be produced within a short period (DeBach and Rosen, 1991). Parasitoids used in biological control programs are typically wasps (Dent, 2000). Many of these are parthenogenetic; therefore, they do not need to mate in order to produce viable offspring.

Pathogens are microscopic organisms that live inside a particular host, causing detrimental effects to its health. There are four main groups of insect pathogens: bacteria, fungi, viruses, and protozoans. Pathogens tend to be host specific or have limited host specificity (Bailey, 1971). Pathogens gain entry into a host through any natural or artificial opening; however, they typically gain entry through the mouth during feeding. In biological control programs, pathogens (microbial control agents) are usually sprayed on the food source of the pest to ensure that they are ingested and cause infection (DeBach and Rosen, 1991). Despite the low risk of non-target effects, there are currently only a few pathogens registered for use in IPM programs.

Historically, predatory coccinellids have been associated with biological control more often than any other predatory insect (Obrycki and Kring, 1998). Predaceous

coccinellids have been used successfully in classical biological control programs since the late 1800s. One of the first and most famous cases occurred in California in 1888, whereby the vedalia beetle, *Rodalia cardinalis* (Mulsant), was imported from Australia to control the cottony cushion scale, *Icerya purchasi* Maskell on citrus crops (Doutt, 1964; DeBach and Rosen, 1991). During the previous summer, a massive outbreak of cottony cushion scale threatened the citrus industry in California. The citrus growers turned to the government for help and an expedition to Australia resulted in the importation of several natural enemies, including the predaceous coccinellid, *Rodolia cardinalis*. Within months of its release, the vedalia beetle became widely established and farmers reported their fields to be entirely clear of cottony cushion scale (Doutt, 1964).

The success of the vedalia beetle in California set the stage for the use of predaceous coccinellids in biological control. As of 2000, predaceous coccinellids were used in 155 attempts to control aphids and 613 attempts to control scale insects (Dixon, 2000). Worldwide, predaceous coccinellids have also been used to control mealybugs and mites (Obrycki and Kring, 1998). The efficacy of these coccinellids has been difficult to determine due to their mobility and polyphagous behaviour (Obrycki and Kring, 1998).

Many endoparasites and pathogens are known to infect predaceous coccinellids, including numerous species of microsporidia, fungi, and hymenopteran parasitoids (Richerson, 1970; Ceryngier and Hodek, 1996). Therefore, the efficacy of coccinellids that are used for biological pest control may be greatly affected by disease and parasitization. Furthermore, coccinellids that are evaluated as candidates for biological

control may be dismissed as ineffective based on poor health that may affect host fitness and contribute toward poor performance.

In North America, the coccinellid *Hippodamia convergens* Guérin-Méneville is often used for augmentative biological control of aphids in greenhouses and on field crops. Billions of *H.* convergens are collected annually from their overwintering sites in California and sold by commercial insectaries throughout the United States and Canada (Obrycki and Kring, 1998). Due to their tendency to disperse immediately following release, *H. convergens* have been ineffective for controlling aphids in the field (Hagen, 1962; Obrycki and Kring, 1998). Furthermore, *H. convergens* are known to host the parasitic wasp *Dinocampus coccinellae* (Shrank) (Sluss, 1968) and the microsporidian *Nosema hippodamiae* (Lipa and Steinhaus, 1959). Despite reports of parasitoids and microsporidia in *H. convergens*, there are currently no screening procedures in place to ensure the health of field-collected beetles before they are sold.

Microsporidia are obligate, intracellular, pathogens that infect a wide range of hosts, from protozoa to humans (Tanada and Kaya, 1993; Mathis, 2000). Insects and arthropods are their most common hosts (Tanada and Kaya, 1993). Microsporidia produce transmissible spores that contain a polar filament, which is used to penetrate a new host cell and inject an infective sporoplasm (Keeling and McFadden, 1998; Mathis, 2000; Bigliardi and Sacchi, 2001). Microsporidioses may be acute (causing quick death of the host) but are often chronic, causing sublethal effects that lower host fitness (Tanada and Kaya, 1993).

Although microsporidia were first reported from *H. convergens* in 1959 (Lipa and Steinhaus, 1959), the means of parasite transmission and the effects of microsporidia on

the life history characteristics of *H. convergens* are not known. The objectives of this study are to examine intra-species transmission of a microsporidian parasite (both vertical and horizontal routes) in *H. convergens* and to quantify the effects of the pathogen on life history characteristics; including larval development, fecundity, and survival.

Chapter 1

Intraspecies Transmission of an Unidentified Microsporidium Infecting the Convergent Lady Beetle, *Hippodamia convergens* Guérin-Mèneville (Coleoptera: Coccinellidae)

1. Introduction

Microsporidian spores may be transmitted both horizontally and vertically (Andreadis, 1987; Tanada and Kaya, 1993). Vertical transmission occurs when spores are transmitted from one generation to the next (Andreadis, 1987; Tanada and Kaya, 1993), typically from the female to her progeny. Transovarial transmission is a form of vertical transmission that occurs when eggs are infected with microsporidia while they are in the ovary, whereas transovum transmission occurs when egg surfaces are contaminated with spores that are ingested by newly-hatching larvae (Andreadis, 1987). Vertical transmission from a male to his progeny is rare as the male gametes are small and do not contribute to the cytoplasm of the zygote (Dunn and Smith, 2001).

Horizontal transmission occurs when a pathogen gains entry to the host through a natural or artificial opening. Microsporidia are most commonly transmitted orally (*per os*) when contaminated food is ingested (Andreadis, 1987; Tanada and Kaya, 1993). Contamination of the host's habitat is another common means of horizontal transmission. Infected insects may release spores into the environment in their feces, when infected hosts die and subsequently decompose, and potentially through any other substance that is excreted by an infected individual (Andreadis, 1987). Uninfected individuals that come into contact with these spores may ingest them and become infected.

Hippodamia convergens are known hosts of microsporidia (Lipa and Steinhaus, 1959; Sluss, 1968). These lady beetles are cannibalistic (Hagen, 1970) and will readily feed on the eggs of other coccinellids if aphids are in short supply. If microsporidian spores are vertically transmitted in *H. convergens*, it is possible for spores to be consumed when infected eggs are cannibalized. Furthermore, *H. convergens* secrete a distasteful coxal fluid when threatened or agitated, a process known as 'reflex bleeding'. Alkaloids in the reflex blood are responsible for the toxic and distasteful properties of the fluid and pyrazines are blamed for the repellent smell produced by the fluid (Ceryngier and Hodek, 1996). There is potential that this fluid may carry microsporidian spores that may be transmitted to uninfected hosts. Another potential means of horizontal transmission is through contact with infected feces.

The objective of this study was to investigate the modes of transmission of an unidentified microsporidium in *H. convergens*. The efficiency of vertical transmission was investigated and the potential for horizontal transmission through contaminated eggs, feces, and coxal secretions were examined.

2. Materials and Methods

Uninfected and microsporidia-infected *Hippodamia convergens* used in this study were isolated from a single shipment of beetles that was purchased from a commercial insectary in July 2004.

H. convergens were reared individually in 120 ml clear, polyethylene cups (Marivac Ltd., QC). A 2.2-cm diameter hole was cut in the side of each cup and a fine mesh screen (80 μm; Bioquip, CA) was affixed to the perimeter. This allowed for air

circulation and prevented the beetles from escaping. Before each use, cups were soaked in a 10% bleach solution (10 min), then rinsed with distilled water and allowed to dry. When in use, cups were also cleaned as needed by wiping out the bottom with a sterile cotton tipped applicator (Puritan Medical Products Co., ME). A 5.5-cm diameter piece of filter paper was used to line the lid of each cup. To prevent cross contamination, instruments were surface sterilized before each use by dipping them in 100% ethanol.

Green peach aphids (*Myzus persicae* Sulzer) were used as food for *H. convergens* in all transmission trials. These were reared on nasturtium (*Tropaeolum minus*) (Dwarf Jewel Mixed, Stokes Seed Ltd., ON) in Sanyo (Model MLR-350H) environmental chambers under controlled conditions (16L:8D; 25±1°C). To ensure that microsporidian spores were not present in aphids used as food in these studies, individuals were periodically collected at random, smeared and examined for microsporidian spores.

2.1 Vertical Transmission:

Eggs were randomly collected daily from 6 microsporidia-infected females for 7 days. Females (30-45 days old) were fed *ad libitum* on a diet of *M. persicae* and provided water through a moistened cotton wick (Crosstex International, NY). Beeltes were maintained in a Sanyo (Model MLR-350H) environmental chamber under controlled conditions (16L:8D; 25±1°C). Eggs were collected daily and smeared individually, then stained with 10% buffered Giemsa (pH 6.9, Sigma Diagnostics) and examined for microsporidian spores by light microscopy. Parent females were also smeared and examined for microsporidian spores to confirm infection. Spore presence in the eggs was used as a sign of successful vertical transmission.

2.2 Horizontal Transmission:

Egg clutches from uninfected females were isolated in polyethylene cups and maintained under controlled conditions (16L:8D; 25±1°C). To confirm that these eggs were uninfected, additional eggs were randomly selected from the same female parent. These were smeared and examined for microsporidian spores. The female parent was also smeared and examined at the end of the trial. Newly-hatched larvae were supplied water daily through a moistened cotton wick and reared to adult on an *ad libitum* diet of *M. persicae*. Following eclosion, adults were isolated in polyethylene rearing cups and from this point on, all work was carried out in a biological safety cabinet (Class II, Baker Company, ME). Before any specimens were examined, the interior surfaces of the cabinet were sprayed with Virox disinfectant (Virox Technologies Inc., ON). Controls were examined before treatments to prevent cross contamination. After the beetles were fed, the interior surface of the cabinet was again sprayed with Virox disinfectant, then exposed to UV light (1 h).

On the day of adult emergence, individual beetles were fed either 5 uninfected eggs (n=30 beetles) or 5 microsporidia-infected eggs (n=31beetles). A fine brush was used to place the eggs carefully on the screen of each rearing cup that already contained an isolated beetle. Eggs were confirmed to be infected by smearing other eggs that were produced by the same female, as well as the parent female at the end of the trial, and examining them for spores. Within 24h, all of the beetles used in the trial had eaten all five eggs that were provided. Distilled water was supplied through a moistened cotton wick.

From the second day on, beetles were fed an *ad libitum* diet of *M. persicae*. Beetles were smeared upon death or at the end of the trial, stained with Giemsa and examined for the presence of microsporidian spores. Presence of spores was taken as evidence of succesful horizontal transmission. A 2x2 χ^2 contingency table (Bailey, 1964) was used to determine significance for 14 day survival between the two groups. Only beetles that survived for a minimum of 48h after eclosion were included in the analysis.

To determine if microsporidian spores were present in coxal fluids that are used for defense, 21 microsporidia-infected beetles were distressed by holding them and applying gentle pressure until a drop of coxal fluid was secreted. This fluid was then smeared directly on a slide, stained with Giemsa, and examined for the presence of spores.

To determine whether microsporidian spores are excreted in the feces, fecal pellets were collected from 3 microsporidia-infected females over 7 days (5 pellets/beetle). Beetles often deposited their fecal pellets on the undersides of the rearing lids; therefore, the lids of individual rearing cups were lined with a piece of filter paper (5.5-cm diameter) that was replaced daily. Fecal pellets were removed from the filter paper using sterilized forceps and were smeared on protein (3-aminopropyltriethoxysilane) treated slides. Smears were stained with Giemsa and examined for microsporidian spores.

3. Results

3.1 Vertical transmission

A total of 245 eggs were collected from 6 infected females over 7 days. Spores were detected in all 245 eggs; therefore, vertical transmission from female to offspring was 100% efficient.

3.2 *Horizontal transmission*

Microsporidian spores were not detected in *H. convergens* fed uninfected eggs (n = 30). However, 26 of the 31 beetles (83.9%) that were fed microsporidia-infected eggs became infected with microsporidia (Table 1). Of the 5 beetles that did not become infected, 3 did not live the full 14 days of the trial. Survival of uninfected and microsporidia-infected beetles over the 14 day trial did not differ significantly (χ^2=1.06, df=1, P>0.05) (Table 1). Microsporidian spores were detected in 4 of 21 (19%) coxal fluid smears; however, spores were not detected in fecal pellets from microsporidia-infected beetles (n = 15 pellets).

Microsporidia were not detected in any of the aphids examined during this study.

4. Discussion

Vertical transmission of this microsporidium was 100% efficient. It is likely that this microsporidium is transovarially transmitted because mature spores were observed within all egg smears. Eggs were not surface sterilized prior to being smeared; therefore, it cannot be determined if microsporidian spores also contaminate the egg surface.

With a 100% vertical transmission rate, microsporidia ensure their survival among a given population as long as the parasite does not cause too great of a burden on the host (Dunn and Smith, 2001). If a hosts dies before it is able to reproduce, the microsporidia that infects it will also die unless they are capable of being transmitted horizontally. Many microsporidia utilize both vertical and horizontal transmission routes to ensure their survival within the host population (Dunn and Smith, 2001).

H. convergens are cannibalistic and will readily feed on their own eggs or larvae, as well as those of other lady beetles if aphids are in short supply (Hagen, 1970). In this study, microsporidia were horizontally transmitted to 83.9% of adult *H. convergens* that were fed microsporidia-infected eggs. This high level of horizontal transmission suggests that the parasite may readily reinfect an infected host (autoinfection) or be transmitted to healthy individuals. Because cannibalism is common among predaceous coccinellids, the results from this study suggest that horizontal transmission is likely to occur when microsporidia-infected eggs are ingested.

In the wild, reflex bleeding by infected individuals may also contribute toward successful horizontal transmission of the microsporidium. Microsporidian spores were found within smear preparations of the coxal fluids from almost 20% of the infected beetles that were examined in this study. Although the technique used to collect coxal fluid was relatively crude, beetles are likely to undergo similar distress in their environment. Because of the technique used, it is possible that some of the spores that were detected originated from the exoskeleton of infected beetles rather than from the fluid itself. Histological studies could help clarify the origin of these spores. Regardless

of the origin, the results suggest that microsporidian spores are liberated from the body and may provide another means of horizontal transmission.

Microsporidian spores were not detected in the fecal pellets of microsporidia-infected *H. convergens*. The absence of detectable spores may be due to the precision of the technique used to detect them. The feces of *H. convergens* contained a variety of excretory materials, including various crystals and aphid exoskeleton that made spore detection difficult by light microscopy. Information from tissue pathology studies may help shed some light on whether or not spores are likely to be transmitted horizontally through infected feces. For example, if spores are routinely observed in the alimentary tract or Malpighian tubules, it is likely that they are excreted in the feces.

Horizontal transmission is most likely to occur when large numbers of beetles are kept in closed quarters where dispersal is restricted, such as in a canvas bag during shipping or following their release in a greenhouse. Beetles that are field-collected from their overwintering sites are unlikely to produce eggs during shipment or storage; however, microsporidian spores may be transmitted through coxal secretions if infected beetles are sufficiently distressed. Increased horizontal transmission due to overcrowding and cannibalism has been demonstrated for the microsporidium *Nosema muscidifuracis* in the pteromalid parasitoid *Muscidifurax raptor* Girault and Saunders (Geden *et al.*, 1995) during superparasitism of the host.

Microsporidia have not been reported from *H. convergens* collected in Canada. However, if infected individuals continue to be collected and redistributed from California, it is possible for this microsporidium to become established in native populations. The microsporidium infecting *H. convergens* has not only a high horizontal

transmission rate (83.9%) when spores are ingested, but it is transmitted vertically with 100% efficiency. Once an infected individual is released, the microsporidium may spread readily from one individual to another.

Although *H. convergens* are purchased for aphid control in Canada, there are currently no quality control measures in place to ensure the health of the beetles used. Microsporidia are known to infect field-collected *H. convergens* that are used for biological control and the results of this study show that these parasites are readily transmitted both vertically and horizontally. Therefore, it is important to implement quality control standards for *H. convergens*, including the routine screening of beetles for microsporidia prior to their release, to prevent the inadvertent distribution of microsporidian pathogens (Kluge and Caldwell, 1992).

Chapter 2

Effects of an Unidentified Microsporidium on Larval Development, Fecundity and Survival of the Convergent Lady Beetle, *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) Used for Biological Control

1. Introduction

In North America, the convergent lady beetle, *Hippodamia convergens* is frequently used for biological control. Billions of *H. convergens* adults are collected annually from their overwintering sites in the Sierra Nevada Mountains in southern California (Obrycki and Kring, 1998). Commercial insectaries then sell these beetles to commercial growers and home gardeners throughout the United States and Canada where they are released in large numbers for aphid control (Obrycki and Kring, 1998). There are currently no screening procedures in place to ensure the health or quality of these beetles before they are distributed and released (van Lenteren, 2003; van Lenteren *et al.*, 2003; Obrycki and Kring, 1998).

H. convergens has been reported as ineffective for aphid control because of their tendency to disperse after they are released in the field (Hagen, 1962; Obrycki and Kring, 1998). The microsporidium *Nosema hippodamiae* is known to infect *H. convergens* (Lipa and Steinhaus, 1959) and although *N. hippodamiae* was first identified in 1959 (Lipa and Steinhaus, 1959), the effects of microsporidia on the life history characteristics of *H. convergens* have not been studied. It is possible that field-collected *H. convergens* used for biological control are infected with microsporidia. Therefore, microsporidioses

may also contribute toward the poor performance that has been noted for *H. convergens* in the field.

The objectives of this study were to investigate the effects of microsporidiosis on life history characteristics of *H. convergens*. Egg hatch, larval development, and adult fecundity and survival of microsporidia-infected *H. convergens* were compared to those of uninfected beetles.

2. Materials and Methods

Hippodamia convergens used in this study were purchased from a commercial insectary. Microsporidia-infected and uninfected beetles were isolated from a single shipment that was received in July 2004.

H. convergens were reared individually in 120 ml clear, polyethylene cups (Marivac Ltd., QC). A 2.2-cm diameter hole was cut in the side of each cup and a fine mesh screen (80 μm; Bioquip, CA) was affixed to the perimeter. This allowed for air circulation and prevented the beetles from escaping. Before each use, cups were soaked in a 10% bleach solution (10 min), then rinsed with distilled water and allowed to dry. When in use, cups were also cleaned as needed by wiping out the bottom with a sterile cotton tipped applicator (Puritan Medical Products Co., ME). A 5.5-cm diameter piece of filter paper was used to line the lid of each cup. To prevent cross contamination, instruments were surface sterilized before each use by dipping them in 100% ethanol.

Nasturtiums (*T. minus*) (dwarf jewel mixed; Stokes Seed Ltd., ON) were grown from seed in Sanyo MLR-350H environmental chambers (16L:8D, 25±1°C). Three-week

old plants were used to rear colonies of green peach aphid (*M. persicae*) (16L:8D, 25°C), which were then fed to *H. convergens* throughout the trials.

2.1 Larval development

Eggs were randomly collected from both uninfected and microsporidia-infected *H. convergens* females (n = 5 females, respectively). Eggs were isolated individually in polyethylene cups and maintained under controlled conditions (16L:8:D; 25±1°C). Newly-hatched larvae were fed an *ad libitum* diet of *M. persicae* and provided distilled water through a moistened cotton wick (Crosstex International, NY) on a daily basis. Eggs and larvae were examined every 6 h and larval instar was recorded. Larvae that died before completing development were smeared, stained with 10% buffered Giemsa (pH 6.9, Sigma Diagnostics), and examined for microsporidian spores by light microscopy. Larvae that completed their development and emerged as adults were sexed. Adults were then smeared, stained with 10% buffered Giemsa, and examined for microsporidian spores. Spore measurements (n = 50) were taken from prepared slides.

Larvae that died during the trial were not included in the analyses for the life stage in which they died or for total larval development. For all analyses, data were checked for normality when relevant. A Mann-Whitney test (Sokal and Rohlf, 1995) was used to determine signifiant differences in development times for eggs, combined larval instars (1-4), pupae, and total larval development. A conservative testing procedure (α = 0.0125) was used to protect against type I errors. A 2x2 χ^2 contingency table (Bailey, 1964) was used to determine significance for adult sex ratios and larval mortality.

2.2 Adult fecundity and survival

Eggs clutches were isolated in polyethylene cups that were maintained under controlled conditions (16L:8D; 25±1°C). Newly-hatched larvae were reared to adult on an *ad libitum* diet of *M. persicae*. Adult females were isolated in polyethylene rearing cups for five days following eclosion on an artificial diet that consisted of ladybeetle and lacewing food (Planet Natural, MT) mixed with pure Nova Scotia honey (10g:10g:1ml distilled water). Water was supplied daily through a moistened cotton wick. From the sixth day on, females were supplied an *ad libitum* diet of green peach aphids.

Females were mated on the sixth day following eclosion by placing a male of unknown age with a virgin female for 24h. Males were then smeared, stained with Giemsa, and examined for microsporidian spores by light microscopy. After the females were mated, the cups were examined daily for eggs. Beetles had a tendency to attach their eggs to the filter paper that lined the underside of the cup lid. When this occurred, the filter paper and eggs were carefully removed and placed into a separate cup. If eggs were laid on the inside of the cup, the female was placed in a new cup so that the eggs were not disturbed. Upon death of the female or at the end of the 90-day trial, females were smeared, stained with Giemsa, and examined for microsporidian spores by light microscopy.

A two sample t-test (Sokal and Rohlf, 1995) was used to determine significance for total mean eggs produced by unifected and microsporidia-infected females. A Mann-Whitney test (Sokal and Rohlf, 1995) was used to determine significance for mean survival, whereas a 2x2 χ^2 contigency table (Bailey, 1964) was used to analyze adult survival.

2.3 Egg viability

Eggs collected during the 90-day fecundity trial were examined daily for larval emergence. Eggs were considered non-viable if they did not hatch after five days. Occasionally, eggs that failed to hatch were cannibalized by newly-emerged larvae. These cannibalized eggs could not be verified as viable, and as a result, the egg hatch data reported herein represents minimum hatch only. A two sample t-test (Sokal and Rohlf, 1995) was used to determine significance and only adult females that produced at least one viable egg were included in the analysis.

3. Results

3.1 Larval development

No significant difference was detected for development times between microsporidia-infected and uninfected eggs ($U = 1590$, $P=0.013$; Table 2). Microsporidia-infected *H. convergens* larvae took significantly longer to develop than uninfected larvae ($U = 372.5$, $P<0.001$; Table 2), and development times for microsporidia -infected *H. convergens* pupae were significantly longer than those of uninfected pupae ($U = 331$, $P = 0.001$; Table 2). Additionally, total development times were significantly longer for microsporidia-infected larvae ($U = 277$, $P<0.001$; Table 2). However, larval mortality did not differ significantly ($\chi^2=0.40$, $df=1$, $P>0.05$) (Table 3).

Sex ratios of microsporidia-infected and uninfected *H. convergens* were not significantly different ($\chi^2=0.11$, $df=1$, $P>0.05$). Sex ratios were 1.06:1 (17♀:16♂) and 1.25:1 (20♀:16♂) for microsporidia-infected and uninfected *H. convergens*, respectively. Microsporidian spores from prepared slides measured 3.6 x 2.4 μm ($n=50$).

3.2 Adult fecundity and survival

During the 90-day trial, cumulative mean egg production of microsporidia-infected females (545.8±92.6 eggs, n=24) was significantly lower than that of uninfected females (928.3±86.4 eggs, n=22) (t = 3.006, df = 44, P = 0.004). Mean egg production for microsporidia-infected and uninfected females was similar until about day 45; however, egg production then declined steadily for microsporidia-infected beetles (Fig.1).

Microsporidia-infected females did not live as long as uninfected females (Fig. 2). Mean survival for microsporidia-infected and uninfected beetles was 64.5±5.6 days (n= 24), and 77.1±4.5 days (n = 22), respectively (U=354.5, P=0.04). At the end of the 90-day trial, 59% (13/22) of the uninfected females were still alive, whereas only 25% (6/24) of the infected females had survived (χ^2=5.50, df=1, P<0.05).

3.3 Egg viability

There was no significant difference between mean egg hatch from microsporidia-infected (47.4±7.6, n=8) and uninfected *H. convergens* (53.61±10.96, n = 6) (t=0.477, df = 12, P>0.05).

4. Discussion

Most microsporidia are thought to be host specific but there are cases where a particular microsporidium is known to infect several hosts (Solter *et al.*, 2005). Two of four species of microsporidia identified from predacious coccinellids have been reported from *H. convergens*. In 1959, *Nosema hippodamiae* Lipa and Steinhaus was observed in the midgut and fat body, whereas Sluss (1968) reported an unidentified microsporidium

in both *H. convergens* and its endoparasitoid, *Dinocampus coccinellae*. In the latter case, it is unclear if the pathogen is *N. hippodamiae* or another microsporidium. Furthermore, *N. tracheophila* Cali and Briggs has been observed in the tracheal epithelium and connective tissues of the seven-spotted lady beetle, *Coccinella septempunctata* L. (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*, *Hippodamia tredecimpunctata* L. and *Myrrha octodecimguttata* L. (Lipa 1968).

N. hippodamiae spores measure 3.3-5.4 x 2.2-2.7 μm (fixed and stained; Lipa and Steinhaus, 1959). Microsporidian spores from *H. convergens* in this study measured 3.6 x 2.4 μm and although these spores are within the range reported for *N. hippodamiae*, they are also similar in size to those of *N. tracheophila* (3.7 x 2.3 μm; Cali and Briggs, 1967). *N. tracheophila*, however, is not known to infect *H. convergens*. Molecular characterization and ultrastructural studies are needed to identify the microsporidium in this study as *N. tracheophila*, *N. hippodamiae* or another microsporidium.

4.1 Larval development

Microsporidia-infected and uninfected *H. convergens* larvae developed faster than *H. convergens* larvae in previous studies when reared at 25°C (Butler Jr.and Dickerson, 1972; Miller, 1992). However, differences in experimental design make it difficult to compare the results of previous studies with this one. For example, different diets were used in earlier studies and larvae were observed less frequently. The latter could explain some of the observed differences in development times for particular life stages, although it is less likely to account for any differences reported for total development.

Microsporidia are known to affect the development of other natural enemies used for biological control. The microsporidium *N. muscidifuracis* impairs the development of the parasitoid *M. raptor* (Boohene *et al.*, 2003) and microsporidia-infected *Trichogramma nubilale* Ertle and Davis and *T. chilonis* Ishii develop more slowly than do uninfected individuals (O'Neil *et al.*, 1998; Sajap and Lewis, 1988; Schuld *et al.*, 1999).

The cause of delayed development in microsporidia-infected insects is not known; however, it has been suggested that infection causes a depletion of energy reserves, less consumption of food, and a lowered efficiency for converting food into energy (as cited by Boohene *et al.*, 2003). Although *H. convergens* larvae used in this study were supplied with *ad libitum* diet of aphids, the number of aphids eaten by each individual was not quantified. Therefore, the cause of delayed larval development in microsporidia-infected *H. convergens* cannot be determined.

In this study, the sex ratios of both microsporidia-infected and uninfected *H. convergens* were not significantly different; suggesting sex ratios were not altered by the microsporidium. Sex ratios for both microsporidia-infected and uninfected beetles were about 1:1, which is consistent with sex ratios reported for *H. convergens* in earlier studies (Smith, 1966; Heimpel and Lundgren 2000). Microsporidia are known to alter the sex ratios of several beneficial arthropods used for biological control (*Metaseiulus occidentalis* (Nesbitt), *Muscidifurax raptor, Phytoseiulus persimilis* Athias-Henriot, and *T. chilonis*) (Bjornson and Keddie, 1999; Schuld *et al.,* 1999; Olsen and Hoy, 2002; Boohene *et al.,* 2003). This is not always the case; however, as the microsporidium *Nosema pyrausta* has no effect on the sex ratio of *T. nubliae* (Sajap and Lewis, 1988).

4.2 Adult fecundity and survival

Lifetime fecundity data for both microsporidia-infected and uninfected *H. convergens* are within the range previously reported by Hagen and Sluss (1966). However, microsporidia-infected *H. convergens* produced fewer eggs per day and fewer eggs overall than did uninfected females. Furthermore, infected females did not live as long as uninfected females. These results are consistent with fecundity and survival data that is reported for other beneficial arthropods infected with microsporidia. Microsporidia reduce the mean fecundity of the parasitoids *Trichogramma evanescens* Westwood (Huger, 1984), *T. chilonis* (Schuld *et al.*, 1999) and *M. raptor* (Boohene *et al.*, 2003). Furthermore, microsporidioses has detrimental effects on the fecundity and survival of the predatory mites *M. occidentalis* and *P. persimilis* (Bjornson and Keddie, 1999; Olsen and Hoy, 2002) and the green lacewing, *Chrysopa callifornica* Coquillett (Finney, 1950).

When microsporidia-infected individuals are contrasted with uninfected ones, the negative effects caused by the microsporidium become more apparent. Based on the mean data collected during this study, uninfected adult females live about 77 days and produce approximately 930 eggs each. When these eggs hatch, the larvae develop to adult within 14.8 days. Conversely, microsporidia-infected females live 64 days and produce approximately 545 eggs, 41% fewer eggs than are produced by uninfected females. When these eggs hatch, infected larvae take 15.4 days to fully develop. Prolonged larval development, observed for microsporidia-infected larvae, may increase their susceptibility to predation.

Natural larval mortality for both uninfected and microsporidia-infected larvae was between 30 and 35%. Based on the data collected from this study, and without taking predation into consideration, individual uninfected female *H. convergens* will produce 300-325 progeny that survive to adulthood, with about 150-165 of those being females. Conversely, individual microsporidia-infected female will produce about 175-190 successful progeny, with about 88-95 of those being females. Therefore, microsporidia-infected females will produce about 41% fewer progeny than will their uninfected counterparts after just one generation.

H. convergens has been reported as ineffective for aphid control in the field (Hagen, 1962; Obrycki and Kring, 1998), primarily because of their habit of dispersing once they are released (Hagen, 1962; Obrycki and Kring, 1998). Efficacy may be further reduced if microsporidia-infected adults are released because fecundity and survival are significantly reduced as a result of infection.

Over the first 40 days of this study, infected beetles produced about as many eggs as uninfected ones. Unfortunately, beetles that are received from biocontrol suppliers are collected from overwintering sites (Obrycki and Kring, 1998) and have already spent a month or two in an active state prior to overwintering (Hagen, 1970). Therefore, when adult *H. convergens* are received from commercial insectaries, microsporidia-infected females may already be at an age where their fecundity has begun to decline.

4.3 Egg viability

Microsporidiosis does not affect egg hatch; however, the majority of females used in this study did not produce viable eggs (32 of 46). This is likely due to insufficient mating periods because each virgin female was confined with a male for 24h. It was later observed in a separate study that a higher proportion of females (8/10) produced viable eggs when they were confined with a male for 72h or longer. Furthermore, females that did not produce viable eggs initially were able to produce viable eggs once they were re-mated. Even if the females used to assess egg hatch had been mated for a longer period, it is unlikely that significant differences would have been observed because percent egg hatch during the larval development trial did not differ significantly.

4.4 Conclusion

Although *N. hippodamiae*, and perhaps other microsporidia, are known to infect field-collected *H. convergens*, there are currently no screening procedures in place to ensure the health or quality of these beetles before they are distributed and released (van Lenteren, 2003; van Lenteren *et al.*, 2003; Obrycki and Kring, 1998). Based on the results from this study, microsporidia adversely affect several life history characteristics of *H. convergens* and may reduce their efficacy when used for aphid control in integrated pest management programs. Field-collected beetles should be screened for pathogens on a routine basis to ensure that they are not infected with microsporidia.

Table 1

Percent infection and survival of *H. convergens* fed uninfected and microsporidia-infected eggs

Uninfected			Infected		
n	% infected	14 day survival	*n*	% infected	14 day survival
30	0.0	27a	31	83.9	25a

a, not significantly different ($P>0.05$)

Table 2

Larval development (days) for microsporidia-infected and uninfected *H. convergens*

	Uninfected				Infected			
	Min	Max	Mean ± SE	*n*	Min	Max	Mean ± SE	*n*
Egg	3.25	3.50	3.32 ± 0.02	51	3.00	3.75	3.27 ± 0.02	51
1st instar	2.25	3.25	2.49 ± 0.04	44	2.00	3.25	2.50 ± 0.05	39
2nd instar	1.50	4.75	1.82 ± 0.11	41	1.50	2.25	1.84 ± 0.04	38
3rd instar	1.75	2.25	1.98 ± 0.03	38	1.00	2.75	2.05 ± 0.05	37
4th instar	2.75	5.00	3.27 ± 0.08	38	3.00	7.00	3.69 ± 0.14	37
Prepupa	0.75	1.25	0.91 ± 0.02	37	0.50	1.25	0.97 ± 0.03	37
Pupa	4.25	4.75	4.46 ± 0.03	36	4.25	4.75	4.54 ± 0.03	33
Total	13.75	17.75	14.76 ± 0.16	36	14.25	18.25	15.40 ± 0.14	33

Table 3

Larval mortality for microsporidia-infected and uninfected *H. convergens*

	Uninfected		Infected	
	n	% mortality	*n*	% mortality
1st instar	51	13.7	51	23.5
2nd instar	44	6.8	39	2.6
3rd instar	41	7.3	38	2.6
4th instar	38	0.0	37	0.0
Prepupa	38	2.6	37	0.0
Pupa	37	2.7	37	10.8
Total	36	29.4	33	35.3a

a, Data for total larval mortality are not significantly different (χ^2=0.40, P>0.05)

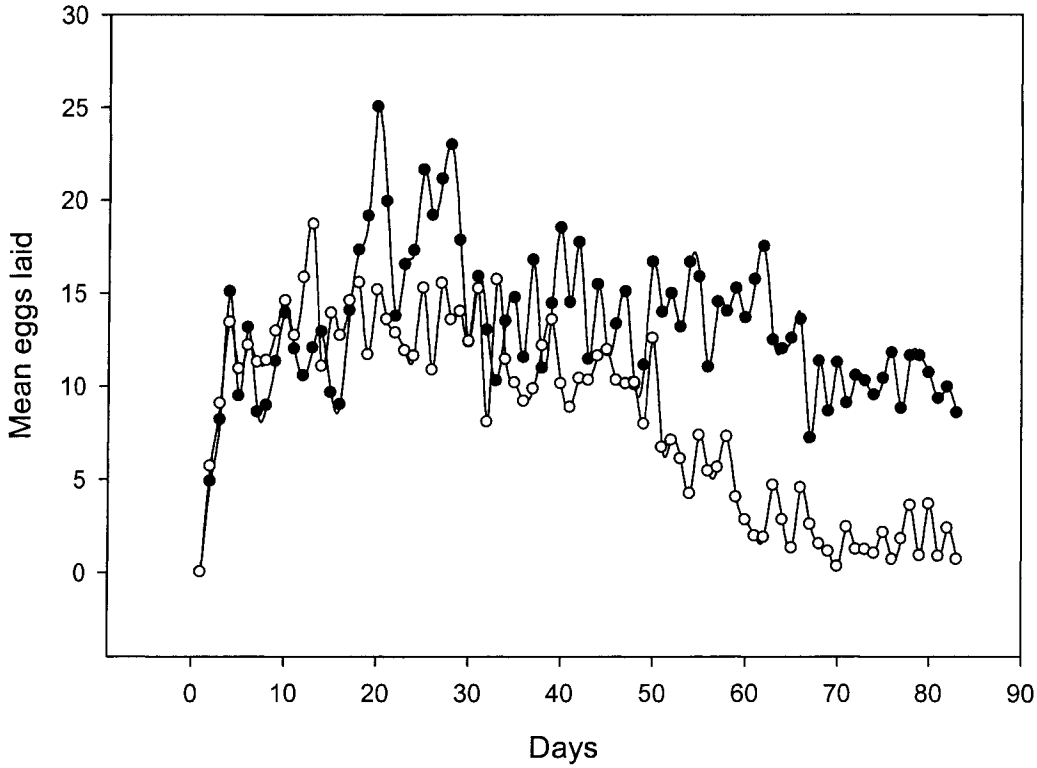

Figure 1: Age-specific oviposition curve showing mean eggs laid per day by uninfected (solid circles) and microsporidia-infected (clear circles) *H. convergens*

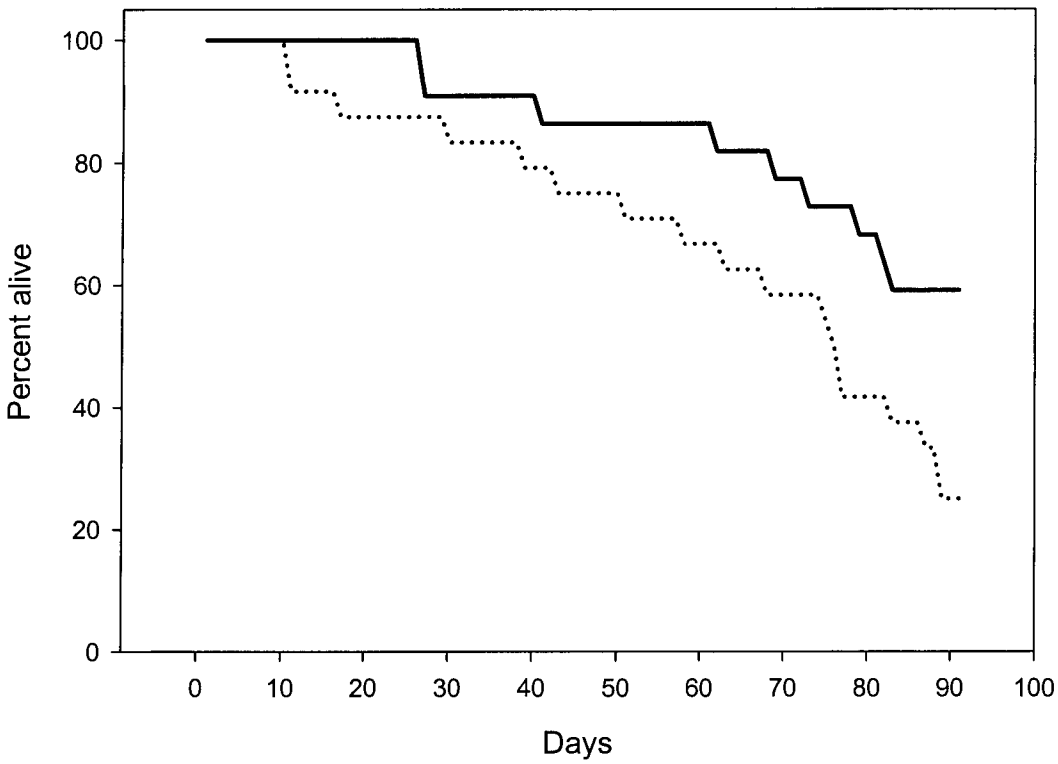

Figure 2: Age-specific survival of uninfected (solid line) and microsporidia-infected (dotted line) *H. convergens* adult females

Conclusion

The unidentified microsporidium in *H. convergens* was vertically transmitted with 100% efficiency. Horizontal transmission was highly successful (83.9%) when microsporidia-infected eggs were ingested by uninfected beetles. Furthermore, spores may be horizontally transmitted through other means. Spores were observed in smear preparations of coxal fluid from infected beetles but it is not clear if these originated from the body surface or if they were present in the fluid itself. Regardless of the origin of these spores, this observation suggests that spores are liberated from the body and these may be transmitted horizontally. Spores were not detected in the feces; however, histological studies are needed to determine which tissues are infected and to shed some light on whether this microsporidium infects the midgut and Malpighian tubules. If this is the case, it is possible that spores are excreted in the feces but went undetected when fecal smears were examined by light microscopy.

Microsporidia-infected *H. convergens* larvae took significantly longer to develop than did their uninfected cohorts. Infected *H. convergens* adult females produced fewer eggs overall and had a higher mortality rate than did uninfected females. Sex ratios for both uninfected and microsporidia-infected *H. convergens* were about 1:1.

H. convergens are considered inefficient as biological control agents by some researchers (Hagen, 1962; Obrycki and Kring, 1998) due to their tendency to disperse immediately upon their release in the field. However, even if beetles remain following their release, the data collected during this study suggests that any microsporidia-infected individuals that remain would not live as long or produce as many eggs as would uninfected individuals.

Although quality control guidelines have been established for many other commercially available biological control agents (van Lenteren *et al.*, 2003), quality control tests focus on natural enemies that are reared in commercial insectaries. Convergent lady beetles are not reared commercially but are collected from their overwintering sites in California; therefore, it is important to implement quality control guidelines to ensure that field-collected *H. convergens* are pathogen free. Ideally, commercially-available *H. convergens* (and other coccinellids) should be reared under controlled conditions in an insectary where it would be possible to control quality and ensure the health of the beetles being sold.

Although *H. convergens* are used for augmentative biological control, the collection and release of this beetle poses problems that are similar to those that are often associated with classical biological control. When a natural enemy is collected from the field, individuals must be quarantined and examined prior to their release to ensure that undetected endoparasitoides or pathogens are not inadvertently released. The formulation and implementation of quality control standards reduces the risk of accidentally releasing exotic endoparasitoids and pathogens along with the natural enemy (Kluge and Caldwell, 1992).

The future of coccinellids as biological control agents is unclear. Though there have been some spectacular successes associated with their use (for example, control of the cottony cushion scale by the vedalia beetle; Doutt, 1964; Debach and Rosen, 1991; Obrycki and Kring, 1998; Dixon, 2000), there have also been failures (for example, displacement of native coccinellids by the multicolored Asian lady beetle, *Harmonia axyridis* (Pallas); Obrycki *et al.*, 2000; Koch, 2003). As with any potential biological

control agent, it is important to fully investigate and document the potential hazards associated with its use.

Although there have been some problems associated with biological control, when used correctly, biological control agents are target specific, self propagating, environmentally friendly, and less expensive than chemical control measures (Tisdell, 1990; Debach and Rosen, 1991; Hagler, 2000; van Emden and Service, 2004). Furthermore, insect pests do not usually develop immunities to their natural enemies, as they often do when chemical control methods are used (van Emden and Service, 2004). Natural enemies are an important part of IPM programs and efforts should be made to ensure that natural enemies are free of pathogens and are efficacious.

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Appendix 1

Giemsa staining procedure

1.	Air dry slides	
2.	Fix in 100% Methanol	10mins
3.	Stain in 10% Buffered Giemsa	90mins
4.	Rinse in tap water	10mins
5.	Dehydrate in following solutions	
	70% Ethanol	3mins
	80% Ethanol	3mins
	90% Ethanol	3mins
	95% Ethanol	3mins
	100%Ethanol	3mins
	Xylene	10mins

6. Cover slips using Permount® histological mounting medium (Fisher Scientific, NJ)

Appendix 2

Recipe for making APTS treated slides

- Soak slides in 2% solution of APTS (3-aminopropyltriethoxysiline) in acetone for 2mins
- Dip 5 times in dH_2O
- Dry in 60^{o} oven