Effects of the microsporidian pathogen, *Nosema adaliae* on the seven-spotted lady beetle, *Coccinella septempunctata* L. (Coleoptera: Coccinellidae)

By

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A Thesis Submitted to

Saint Mary's University, Halifax, Nova Scotia for the

Degree of Master of Science in Applied Science

April 2016, Halifax, Nova Scotia

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Table of Contents

Contents

List of Tables

List of Figures

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Abstract

Lady beetles play an important role in the regulation of aphids and other soft-bodied insects in nature. A noticeable decline in lady beetle populations in North America in recent years has become a significant cause for concern in agriculture systems. The objective of this study was to gain knowledge about the effects of the microsporidium *Nosema adaliae* from the two-spotted lady beetle *Adalia bipunctata* L. on larval development and mortality, sex ratio, and adult longevity and fecundity of the seven-spotted lady beetle, *Coccinella septempunctata* L. Vertical transmission of the pathogen also was examined. Spores were detected in the majority of smear preparations of individuals that were fed microsporidia-infected eggs. Larval development (days) between the control and treatment groups did not differ significantly. Although larval mortality was high for control and treatment larvae, mortality for control and treatment larvae did not differ significantly. Sex ratios differed significantly between the control and treatment groups; however, differences in fecundity and adult longevity were not observed.

Date: 07 April 2016

Acknowledgements

I would like to thank my supervisor, Dr. Susan Bjørnson for giving me this opportunity to undertake this research under her supervision. I would like also to express my appreciation to her for her guidance during the research of my study. Without her valuable assistance, this work would not have been completed. I would also like to thank my lab colleagues Thomas Steele and Kyle Whalen, for their support and cooperation.

1.0 Introduction

Biological control is the use of living organisms to decrease pest population densities or to reduce the economic and damaging impacts of specific pest organisms. Biological control of invertebrate pests can be achieved using predators, parasitoids and pathogens. A common aim of biological control is to promote natural enemies that are already present in the environment, by making conditions favorable for their survival, including the manipulation of environmental conditions and by avoiding the application of chemical pesticides that may reduce the number of beneficial species (Frank and Russell, 2012).

There are three main types of biological control: augmentation, conservation and classical. Augmentation is a form of biological control that involves the manipulation or the supplemental release of natural enemies to make them more efficient at regulating pest populations (DeBach, 1964). This type of control is applied commercially in cropping systems worldwide (van Lenteren and Bueno, 2003) and involves the mass production and periodic release of natural enemies to achieve adequate pest control (Pedigo and Rice, 2006; DeBach, 1964). Conservation biological control focuses on the preservation of existing natural enemies within an ecosystem by reducing the factors that interfere with their survival and by providing resources that beneficial species require to thrive in the local environment (Pedigo and Rice, 2006; Landis and Orr, 2013). Classical biological control uses exotic natural enemies to control 'introduced' or 'non-native' pest populations in areas where the pest species did not previously exist. However, introduced species are often considered 'invasive' when they cause harmful effects in the area where they have been introduced. The

control of introduced, or intruder, species has been recognized as fundamental to the preservation of biodiversity (van Lenteren, 2012). This approach has involved the release of insect parasitoids and predators for weed control and insect pest control (Eilenberg et al., 2001). In general, biological control agents offer environmentally friendly, safe and cost-effective options for pest management (van Lenteren, 2012).

The use of mass-produced or field-collected predators, parasitoids and pathogens for biological pest control is considered to be a successful alternative to chemical pesticides, resulting in higher annual profits for farmers (van Lenteren, 2012). Biological control is often effective at keeping pests at low population levels; therefore, these pests do not cause significant economic damage to crops or livestock (DeBach and Rosen, 1991).

The earliest known record of using natural enemies for pest management dates back to the fourth-century in China. The ancient Chinese observed that ants were effective predators of many citrus pests, so farmers moved nests into their orchards (McCook, 1882; Huang and Pei, 1987; Landis and Orr, 2013) and used interconnecting bamboo rods as bridges to facilitate movement between trees. This method encouraged the ants to provide long-term pest control by permitting them to move freely among the tree canopies (Huang and Pei, 1987). In 1888, the most successful case for a biological control in commercial agriculture in North America involved the introduction of the Australian ladybird, (*Rodolia cardinalis* Mulsant) into California to control the cottony-cushion scale (*Icerya purchasi* Maskell) on citrus trees. One hundred years after its introduction, this beetle it is still effective for scale insect control. This amazing result led to the widespread concept of introducing natural

enemies, especially lady beetles, for biological pest control (Dixon, 2000).

1.1 Microsporidia

Microsporidia are a large group of microbial eukaryotes that are obligate intracellular parasites of other eukaryotes (Keeling and Fast, 2002). Microsporidia infect both invertebrate and vertebrate hosts, including fish, birds, amphibian, reptiles, and mammals (Tanada and Kaya, 1993) and some microsporidia are known to cause infections among immunocompromised humans (Garcia, 2002). Most microsporidia have a rather narrow host range. For example, microsporidia that infect invertebrates tend to be limited to hosts of closely related invertebrate species (Tanada and Kaya, 1993). In the middle of the nineteenth century, the first record of a microsporidian pathogen from insects was described from the silkworm (*Bombyx mori* L.) from southern Europe (Keeling and Fast, 2002). Since then, microsporidia have been reported from several beneficial insects, such as lady beetles and honeybees (Bjørnson and Oi, 2014).

Microsporidia are common pathogens of lady beetles. *Nosema hippodamiae* was described from the convergent ladybird beetle, *Hippodamia convergens* Guérin-Méneville collected in California (Lipa and Steinhaus, 1959) and two species of microsporidia (*Nosema tracheophila,* and *Nosema coccinellae*) have been described from the seven-spotted lady beetle, *Coccinella septempunctata,* (Cali and Briggs, 1967). *Nosema tracheophila* infects the tracheal epithelium, haemocytes and connective tissue of *C. septempunctata* (Cali and Briggs, 1967) and *N coccinellae* infects the midgut epithelium, Malpighian tubules, gonads, nerves and muscle tissues of numerous beetles including *C. septempunctata* and *A. bipunctata* (Lipa, 1968; Lipa

et al., 1975).

Insects infected with microsporidia may show external symptoms that include a change of colour, size, and activity. For example, infected larvae may show a delay in growth or development, be reduced in size prior to death, have reduced activity levels, or affect the ability of larvae to digest food (Tanada and Kaya, 1993). Other symptoms associated with infection may include abnormal feeding, irregular growth, incomplete metamorphosis, deformed pupae and adults, and lowered fecundity (Kluge and Caldwell, 1992). The pathogen *Nosema apis* affects the immunity of honeybees and this is thought to be one of the factors that has caused a recent decline of honeybee populations, a condition known as 'colony collapse disorder'. This phenomenon has been documented as early as 1869 (Tanada and Kaya, 1993).

Microsporidia produce highly resistant spores that are able to survive outside the host (Garcia, 2002). All microsporidia have a unique, highly specialized structure called the polar tube. Environmental stimulation causes an increase in osmotic pressure within the spore and this causes the polar tube to rapidly discharge out of the spore, piercing the host cell membrane to inject the sporoplasm (the infectious stage of the pathogen) into the host cell (Xu and Weiss, 2005). Spore morphology may be helpful for differentiating between species of microsporidia. Most species produce oval or pyriform spores but some microsporidian species produce spores of an unusual rod-like or spherical shape. Microsporidian spores are transmitted to insects through three infection routes: oral, cuticular, and ovarial. Transmission by the oral and cuticular pathways results in horizontal transmission (from one infected individual to another within a population). Vertical transmission (from parent to progeny) is achieved

through the ovarial portal (Tanada and Kaya, 1993).

1.2 Lady beetles

Lady beetles (Family Coccinellidae) are also known as ladybugs or ladybirds. Depending on the species, a single lady beetle can eat as many as 5000 aphids during its lifespan (Bessin, 2013). Lady beetles are a very successful group of beneficial insects that are often used in biological control because both adult and larvae are predaceous. Some lady beetle species feed on scale insects, aphids, mites, adelgids, whiteflies, ants, lacewings, lady beetle larvae, larvae of the alfalfa weevil, and psyllids (Dixon, 2000). Frank and Russell (2012) distinguish lady beetles according to the way they feed. Beetles of the genera *Hippodamia*, *Coccinella*, and *Harmonia* are large, and tend to develop quickly. They are also fast moving, and lay their eggs in clusters. Lady beetles that feed on scale insects are typically smaller and develop more slowly. They live longer and lay their eggs singly.

The advantages of using lady beetles for biological pest control has been known for centuries. Lady beetles were praised for their predacious feeding behaviors as far back as the 1800's when it was suggested that lady beetles could be used to control pests in hot houses (DeBach, 1964). Today, numerous species of lady beetles are commercially available for the control of aphids and other pests on agricultural and ornamental crops (van Lenteren, 2003).

In North America, the convergent lady beetle, *Hippodamia convergens* is commercially available for aphid control in home gardens and on agricultural crops. Millions of *H. convergens* are collected each year from their overwintering sites in the Sierra-Nevada Mountains of California. They are then sold by commercial insectaries

throughout the United States and Canada. This practice of collecting and redistributing *H. convergens* began more than 100 years ago in California. At that time, *H. convergens* were provided to local farmers for distribution among their cantaloupes, prunes, apples, pears and vegetable crops (Carnes, 1912). The practice of collecting and redistributing *H. convergens* continues today, despite evidence that suggests that overwintering *H. convergens* are not always effective for aphid control (Bjørnson, 2008).

Some lady beetles have been introduced for biological pest control and have been ineffective. The introduction and distribution of *Coccinella septempunctata* in North America is thought to have contributed to a decline of several native coccinellid species, including *A. bipunctata*. The introduction of such species may result in negative ecological effects and may threaten biodiversity (Minchin, 2010).

Lady beetles have very few natural predators because of the bitter tasting alkaloids they excrete as a defense mechanism when disturbed. The few predators of lady beetles include several bird species and some ants that feed on aphid honeydew. The latter are extremely protective of the aphids and will attack anything threatening their honeydew supply (Hodek and Honěk, 1996).

Predaceous lady beetles differ in size, color, patterns on their fore wings (elytra), and preferred prey (Tavares et al., 2002). Lady beetles may be oval or hemispherical and can be a variety of colours, including red, yellow, orange or black (Bessin, 2013).

The life cycle of aphidophagous lady beetles begins with eggs that are often laid in clusters. Newly hatched larvae feed on aphids. There are four larval instars,

followed by a pupal stage and the adult (Dixon, 2000). The eggs of most lady beetles are yellow or orange and are often laid on stems and the undersides of leaves for protection. Larvae are alligator-like and are often gray or black with orange or yellow spots (Gray, 2005). This study will focus on the seven- spotted lady beetle (*Coccinella septempunctata*).

1.2.1 Seven-spotted lady beetles, Coccinella septempunctata

Seven-spotted lady beetles are generally 7-8 mm in length with white or pale spots near the pronotum. These dome-shaped beetles have a predictable 7 black spots on their elytra in a distinctive pattern (University of California, 2014). *Coccinella septempunctata* larvae hatch from eggs that are generally laid directly on plant foliage. Larvae develop over several days, depending on the availability of aphids, and pupal development can from 3 to 12 days, depending on environmental temperature. Beetles emerge in spring or early summer to feed on aphids before laying eggs. Each female may lay from 200 to more than 1000 eggs over a one to three month oviposition period. The females usually deposit their eggs in clusters near aphids on the underside the leaves and stems. In Canada, *C. septempunctata* may have 1-2 generations per year. They mainly prey on aphids and other similar scale insects, but adults will survive on pollen and other food sources when aphids are scarce (Bauer, 2013). *C. septempunctata* is a non- native coccinellid that was first introduced into North America from Europe in 1951 for aphid control in the United States (Obrycki et al., 2000; Bauer, 2013). Seven-spotted lady beetles have been used frequently for aphid control in apple orchards and it is the most common lady beetle used for aphid control in Egypt (Minchin, 2010; Mahyoub et al., 2013). Adults are known to eat more than

100 aphids each day (Mahyoub et al., 2013) and the presence or absence of *C. septempunctata* in the local environment depends on aphid availability (Bauer, 2013). The invasion of two exotic lady beetle species, *C. septempunctata* and *Harmonia axyridis* (Pallas) is well documented in North America (Takizawa and Snyder, 2012).

1.2.2 Two-spotted lady beetles, Adalia bipunctata

Two-spotted lady beetles are tree-dwelling species (Omkar and Pervez, 2005) and are one of the most common aphid predators in Europe, North America and central Asia (Jalali et al., 2010). *A. bipunctata* were commercialized for aphid control in Europe in 1999 and were made available in North America shortly thereafter (De Clercq et al., 2005). Early attempts at establishing the two- spotted lady beetle for pest control was unsuccessful until *A. bipunctata* eggs and larvae were used for control of the rosy apple aphid, *Dysaphis plantaginea* (Passerini) in Switzerland (Pervez and Pervez, 2005). In some cases, the successful establishment of *A. bipunctata* for biological control is associated with the combined release of the aphid midge, *Aphidoletes aphidimyza* Rondani, and the marmalade hoverfly, *Episyrphus balteatus* (de Geer). Another strategy involved the use of *A. bipunctata* against the linden aphid, *Eucallipterus tiliae* L., infesting lime trees, *Citrus aurantifolia,* in France (Pervez and Pervez, 2005). The colorization of *A. bipunctata* is controlled by multiple alleles (Hodek and Honěk, 1996) and there are two main forms: melanic and non-melanic. Melanic forms are predominantly black with (four) red spots on the elytra, whereas non-melanic forms are red with two black spots, one on each elytron. The prevalence of melanic and non-melanic *A. bipunctata* is dependent on geographical location and light intensity is the main factor that determines melanic frequency. Melanic *A.*

bipunctata are located in areas of low sunshine because the bodies of melanic morphs have a higher temperature than the non-melanic beetles (Brakefield and Willmer, 1985; De Jong et al., 1996).

The life cycle of *A. bipunctata* is similar to that of other lady beetle species (Dixon, 2000). The life cycle begins with eggs and that are laid in clusters. Aphidophagous *A. bipunctata* typically lay their eggs in clusters whereas coccidophagous lady beetles lay their eggs singularly (Dixon, 2000). Two-spotted lady beetles tend to lay their eggs at sites based on prey availability rather than prey suitability (Fréchette et al., 2006). These beetles feed primarily on aphids but they also eat coccids and diaspids (scale insects) as alternative prey and feed on pollen when aphids are absent or scarce (Hemptinne and Desprets, 1986). Larval development includes four instars, and adult beetles are soon ready for mating once they emerge from the pupae (Dixon, 2000).

Nosema adaliae is the only microsporidium that has been described from field- collected *A. bipunctata.* This pathogen, found in beetles from Nova Scotia, prolonged larval development but had no effect on adult fecundity, longevity or sex ratios. (Steele and Bjørnson, 2012). The aim of my research is to examine the effects of the microsporidian pathogen *Nosema adaliae,* from *Adalia bipunctata* on larval development, mortality, adult fecundity, longevity, and sex ratios of *Coccinella septempunctata*. Vertical transmission of the pathogen will also be examined. *Coccinella septempunctata* is host to other microsporidia, *N. tracheophila* and *N. coccinellae,* and because microsporidia are known to infect closely related hosts, it is likely that *N. adaliae* may also infect *C. septempunctata*.

2.0 Materials & Methods

Uninfected *C. septempunctata* adults used in this study were collected during the summer of 2014 from rose bushes near Saint Mary's University campus (Halifax, Nova Scotia). Beetles were reared in the laboratory and examined by smearing a sample of eggs and larvae from each parent group to ensure that they were free of microsporidia and other pathogens. Eggs that were fed to *C. septempunctata* during this study originated from established colonies of uninfected and *N. adaliae*-infected *A. bipunctata,* maintained in our laboratory. *Coccinella septempunctata* and *A. bipunctata* adults were reared individually in 120 mL clear polyethylene cups with tight- fitting yellow lids. A hole (2.2-cm diameter) in the side of each cup was covered with fine mesh screen to permit air circulation. Both *A. bipunctata* and *C. septempunctata* were maintained under controlled conditions (16:8 L:D; 25°C:20°C) within environmental chambers (Sanyo MLR-350H) and were monitored daily.

Each cup contained a cotton wick (Crosstex International, NY) that was moistened with water daily and artificial diet. The latter consisted of one part honey mixed with one part diet (Lacewing & Ladybug Food, Planet Natural, MT). A small amount of diet was spread on the wall of cup as needed and replenished every 2-4 days. Males and females were maintained in separate cups until they were mated. Mating pairs (a single male and female) were confined within a single cup where they were provided an abundance green peach aphids, *Myzus persicae* (Sulzer), which are essential for oviposition. Aphids were reared on nasturtium (*Tropaeolumminus* L., Dwarf Jewel Mixed; Stokes Seed Ltd., ON) that had been grown in isolated chambers. Because beetles tended to lay their eggs on the underside of each cup lid, each lid was

lined with a 55-mm circular piece of filter paper so that the eggs could be easily removed. Following each use, rearing cups were washed, drenched in a 10% bleach solution (10 min), rinsed, and air- dried. Instruments (forceps, spatulas and paint brushes) that were used for transferring eggs, handling larvae, and feeding were sterilized in 70% ethanol to avoid contamination.

2.1 General experimental procedure

Uninfected *C. septempunctata* larvae used in the experimental trials were obtained from 10 mating pairs. Eggs that were fed to these larvae originated from *A. bipunctata* mating pairs (10 uninfected and 10 *N. adaliae*-infected). Eggs produced by each mating pair were collected daily and isolated in polyethylene cups. The infection status of all eggs and larvae used in the following trials were confirmed by smearing cohort eggs and larvae on microscope slides and examining stained specimens for microsporidian spores. *N. adaliae* is transmitted vertically with 100% efficiency; therefore, the proportion of infected eggs produced infected *A. bipunctata* was 100% (this was confirmed by light microscopic examination of cohort eggs and parent adults at the end of the trial). In the case of *C. septempunctata*, cohort eggs and larvae were routinely examined for microsporidian spores, as were adults at the end of the trial. Smear preparations were air-dried, fixed in methanol (10 min), stained in 5% Giemsa (pH 6.9; 2 h), rinsed in tap water (10 min), and treated to a series of ethanol in ascending concentration (70%, 3 min; 80%, 3 min; 90%, 3 min; 95%, 3 min; and absolute ethanol, 3 min). Slides were finished in xylene (10 min) and mounted in Permount (Fisher Scientific). Specimens were examined for the presence of microsporidian spores by light microscopy (40X magnification).

Test larvae were reared individually in clear Petri dishes (47-mm diameter, Millipore Corp., MA). The lid of each dish was equipped with a 2-cm hole that had been covered by mesh. The age of uninfected *C. septempunctata* test larvae used in the following trials were between one and two days old.

2.2 Effects of Nosema adaliae – pathogen dose response

The purpose of this trial was to determine the effects of pathogen dose on *C. septempunctata* larval development and survival. Test larvae were divided into four treatment groups and individuals from each group were fed four *A. bipunctata* eggs (uninfected and/or microsporidia- infected, depending on treatment; Table 1). Larvae fed uninfected *A. bipunctata* eggs served as a control.

Table 1. Number of uninfected and *Nosema adaliae*-infected eggs fed to *Coccinella septempunctata* (Trial 1)

	n	Uninfected *A. bipunctata* eggs	Infected *A. bipunctata* eggs
Control	36	4	0
Treatment 1	36	3	1
Treatment 2	36	2	2
Treatment 3	36	0	4

On the first day of the trial, a 6-mm disc of moistened filter paper containing 4 *A. bipunctata* eggs were placed in the centre of the Petri dish. Larvae were given two days to eat all 4 eggs and were provided only water during this period. After the two days had lapsed, larvae that had eaten all of the eggs were provided a diet of aphids. The larvae that did not eat all 4 eggs were discarded and excluded from the data analysis. Larval development and mortality were observed daily.

Six larvae (for each of the four treatment groups) were set up daily for 6 days (36 larvae per treatment, total *n*=144). This trial was not repeated because the majority of the test larvae died during the trial. With one exception, all test larvae developed into pupae but all died before they eclosed as adults. Because the objective was to observe the effects of the pathogen in both larvae and adults, and mortality in this trial was nearly 100%, this trial was not repeated. Smear preparations of these larvae were stained and examined by light microscopy for the presence of microsporidian spores.

2.3 Effects of Nosema adaliae on larval development

Following the death of the majority of test larvae in the initial trial, a second trial was undertaken to examine pathogen effects on *C. septempunctata* larvae. Test larvae used in this trial were two days old and each was provided either a single uninfected or microsporidia-infected *A. bipunctata* egg (Table 2). Larvae that consumed the egg within 24 hours were provided aphids daily throughout the remainder of the trial. Larval development and mortality were observed daily. For each treatment group, 10 larvae were set up daily for a total of 4 days (40 larvae per treatment). This trial was repeated (one trial was conducted in January 2015 and the second trial in May 2015).

Table 2. Number of uninfected and *N. adaliae*-infected eggs fed to *Coccinella septempunctata* (Trial 2)

	n (per trial)	Uninfected *A. bipunctata* eggs	*Nosema adaliae*-infected *A. bipunctata* eggs
Control	40	1	0
Treatment 1	40	0	1

Larvae, pupae, and adults that died during the trial, were smeared, stained with Giemsa and examined by light microscopy for the presence of microsporidian spores. Mean larval development time (days) was calculated. Data from larvae that did not eat the *A. bipunctata* egg after 24 h were not included in the analyses (n=32 larvae from the control, n=30 from the treatment group). Data from both trials were pooled. A t-test was used to determine significance in the duration of larval development (days). A chi-square test was used to analyze differences in larval mortality between groups.

2.4 Effects of Nosema adaliae on adult Coccinella septempunctata

Adult *C. septempunctata* that emerged from the above trial (29/68 adults from the control group and 18/68 from the treatment) were sexed two days after eclosion. Female beetles from the control (n=13) and treatment groups (n=15) were mated with uninfected male beetles from laboratory-reared colonies. These mated females were used in 60-day fecundity and longevity trials. Mating pairs were isolated within polyethylene cups and males were removed once the females began to lay eggs (between 4 to8 days). Females were fed a diet of aphids and any eggs that were laid were removed and counted daily. Some of the females died before laying eggs. At the end of trial, female and male beetles that were used for mating pairs were smeared and examined for microsporidian spores.

2.4.1 Adult fecundity and longevity

Female *C. septempunctata* in this trial originated as larvae that were fed one uninfected or microsporidia-infected *A. bipunctata* egg (see section 2.4). During the 60-day trial, the number of eggs produced by each female was recorded. All females that died

during the 60 days, as well as those adults that survived to the end of the trial, were smeared, stained, and examined for microsporidian spores by light microscopy to determine their infection status.

2.4.2 Vertical transmission

C. septempunctata eggs (a maximum of 10 per female per day) were randomly selected from each mating pair for microscopic examination for the duration of the 60-day trial. A total of 2984 and 1290 *C. septempunctata* eggs were examined from the control and treatment groups, respectively. Presence or absence of spores was used to determine daily percent vertical transmission (number of eggs that contained microsporidia spores/total number of eggs, multiplied by 100).

3.0 Results

Male and female *C. septempunctata* parents used to produce uninfected test larvae for the experimental trials were not infected with microsporidian spores. All of the eggs that were collected and examined from these beetles were also microsporidia-free. Microscopic examination of *A. bipunctata* mating pairs and eggs, collected from these parents, confirmed that the *A. bipunctata* eggs fed to *C. septempunctata* test larvae in this study were either uninfected or infected with *N. adaliae* (used in control and treatment groups, respectively). At the end of first trial, an unidentified microorganism was detected in the many of the larval smear preparations. Based on size, these microorganisms are bacteria, likely belonging to the genus *Bacillus*. The total number of infected larvae with bacteria per treatment group was 19/21 infected (control) and 8/23, 11/26 and 6/25 for treatments 1 to 3, respectively.

3.1 Effects of Nosema adaliae – pathogen dose response

During the first trial, when *C. septempunctata* larvae were fed a diet of 4 *A. bipunctata* eggs, mortality for test larvae in both the control and treatment groups was near 100% (Table 3). Larvae were unable to complete their development, with the exception of one individual from treatment 3 that pupated successfully and emerged as an adult. Larval mortality in the control and two of the treatment groups was 100%, whereas the mortality of larvae from treatment 3 was 95.2%.

Table 3. Development (days) and mortality (percent) of *Coccinella septempunctata* that consumed four *Adalia bipunctata* eggs as first-instar larvae (Trial 1)

	n	Development Mean ± SE (days)	Larvae	Pupae	Adults	Mortality (%)
Control	21	26.0 ± 1.3	19	2	0	100
Treatment 1	23	25.7 ± 1.4	18	5	0	100
Treatment 2	26	27.7 ± 1.1	23	3	0	100
Treatment 3	25	26.6 ± 1.8	20	4	1	95.2

3.2 Effects of Nosema adaliae on larval development

At the end of the 60-day trial, 2 of 68 larvae in the control group were infected with microsporidian spores (2.9% infection; Table 4). Microsporidian spores were detected in the majority of smear preparations of larvae from the treatment group (58.8% transmission).

Table 4. Percent uninfected and microsporidia-infected *A. bipunctata* larvae under laboratory conditions

	n	Uninfected larvae	Infected larvae	Uninfected larvae (%)	Infected larvae (%)
Control	68	66	2	97.0	2.9
Treatment	68	28	40	41.1	58.8

Development (days) of control and treatment *C. septempunctata* larvae did not differ significantly (F=1.67; *df* =134; $p > 0.05$; Table 5). Larval mortality did not differ significantly between the control and treatment groups (χ^2=3.841; d*f*=1; p=0.5). In the control group, 18 larvae died before they developed into pupae, and 20 pupae died before they eclosed as adults. A total of 30 beetles emerged. For the treatment group, 28 larvae died along with 18 pupae and a total of 22 adults emerged.

Table 5. Development (days) and mortality (percent) of *Coccinella septempunctata* larvae that consumed one *Adalia bipunctata* egg (Trial 2)

	n	Mean ± SE	Larvae	Pupae	Adults	Mortality
Control	68	17.8 ± 0.8	18	20	30	55.9
Treatment	68	17.4 ± 0.9	28	18	22	64.7

3.3 Sex Ratio

Sex ratios were 13:16 (female: male) and 15:3 for the control and treatment group, respectively. These sex ratios differed significantly ($\chi^2 = 6.837$, *df* =1, $p = 0.05$) (Table 6).

Table 6. Sex ratio, fecundity, and longevity data for uninfected and microsporidia-infected *Coccinella septempunctata* under laboratory conditions

	n	Fecundity Mean ± SE	P-value	Longevity (Days) Mean ± SE	P-value
Control	6	497.33 ± 92.62		58.33 ± 18.63	
Treatment	4	322 ± 138.96	0.328	43.75 ± 18.74	0.440

3.4 Effects of Nosema adaliae on adult Coccinella septempunctata

3.4.1 Adult fecundity and longevity

Females from the treatment group that were not infected with microsporidia, and the two infected females from the control group at the end of the 60-day trial were excluded from the analysis. An age-specific oviposition curve (mean eggs/day) was constructed for beetles in each of the control and treatment groups (Figure 1). Fecundity of *C. septempunctata* females from the control and treatment groups did not differ significantly (F = 0.527, *df* = 8, *p* = 0.328), nor did the longevity among beetles of these groups (F = 0.837, *df* = 8, *p* = 0.440) (Table 6).

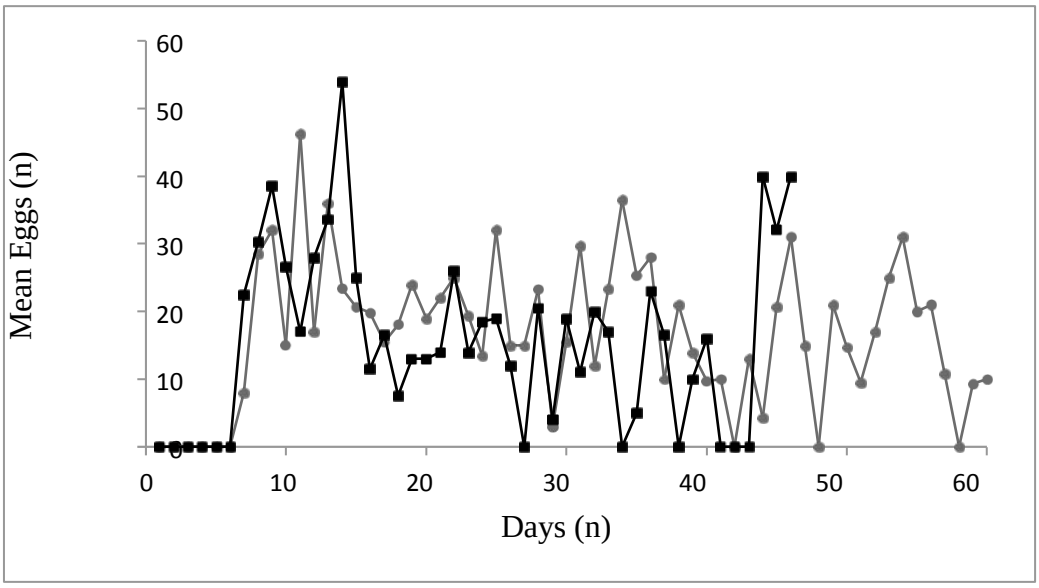

Figure 1. Age specific oviposition curve (mean eggs produced per day) of uninfected and *Nosema adaliae*-infected *Coccinella septempunctata* females during a 60-day trial. Gray line with circles, control; black line with square, treatment.

3.4.2 Vertical transmission

Most eggs produced by the *N. adaliae*-infected females were free from the pathogen from the first day until the seventh day of the trial, with the proportion of infected eggs increasing until 100% infection was reached on Day 13 (Figure 2). Vertical transmission for the lifetime of each of the four females observed during the trial was: 2.5%, 37.2%, 65%, and 98%.

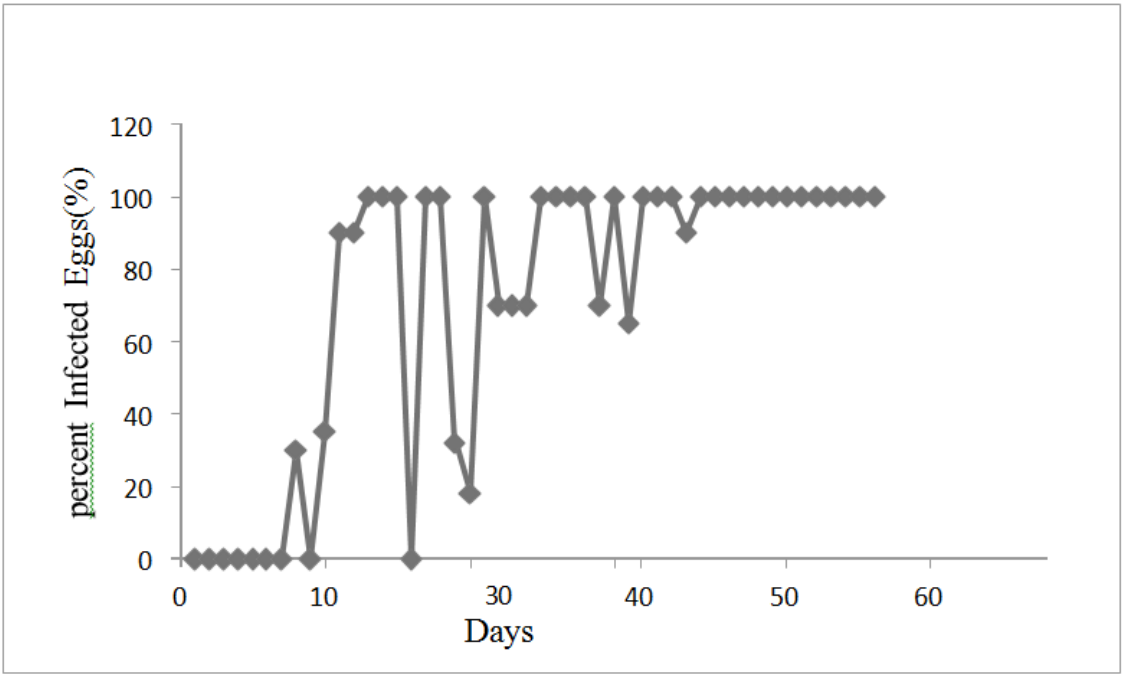

Figure 2. Vertical transmission (percent eggs infected per day) of microsporidia from *Coccinella septempunctata* that were infected as first-instar larvae

4.0 Discussion

Beneficial insects, including lady beetles, play an important role in the natural environment and in agriculture by keeping pest numbers below damaging levels. This decreases the need for insecticides for crop protection (Lee-Mader et al., 2014). Mass reared insects used for biological control are often subjected to high population densities and temporary periods of starvation. Such stresses increase disease

transmission and lead to high disease prevalence that may result in the loss of entire colonies (Vega and Kaya, 2012). Microsporidia are cryptic pathogens that cause chronic and debilitating disease, and these pathogens may go unnoticed in mass rearings unless individuals are examined routinely for microsporidian spores (Kluge and Caldwell, 1992; Bjørnson and Oi, 2014).

There are factors other than disease that may lead to a decrease in the population density of native lady beetle species. For example, the introduction of exotic lady beetle species, such as seven-spotted lady beetles and multicolored Asian lady beetles that aggressively compete for food, may result in the displacement of native species. Losing native lady beetle populations could impact pest populations, both in home gardens and in large agricultural operations. A decrease in lady beetles that feed on aphids and other pest insects could result in considerable damage to food and ornamental crops (Lee-Mader et al., 2014).

4.1 Effects of Nosema adaliae – pathogen dose response

The alkaloids present within lady beetle eggs may have caused the high larval mortality observed during the first trial when *C. septempunctata* larvae were fed four *A. bipunctata* eggs. There is variation in type and concentration of alkaloids within the eggs of various coccinellid species (Smith and Gardiner, 2013) and these differences may affect larval development. For example, *Coleomegilla maculata* (De Geer) and *Olla v-nigrum* Mulsant larvae were unable to complete development when they were fed a diet of *Harmonia axyridis* (Pallas) eggs. The inability of these beetle larvae to develop normally was attributed to the alkaloids present in the *H. axyridis* eggs. On the other hand, *H. axyridis* larvae are able to complete

development when fed a diet that consists of *Coleomegilla maculata* and *Olla v-nigrum* eggs (Smith and Gardiner, 2013). In nature, *C. septempunctata* prefer to eat their own eggs than those of *A. bipunctata* (Turnipseed et al., 2015) and this preference may be due to the types and concentrations of alkaloids within the eggs.

Alkaloids from *A. bipunctata* eggs may be toxic to some coccinellid species, including *C. septempunctata*. In a previous study, *C. septempunctata* larvae did not complete development when fed a diet of *A. bipunctata* eggs (Hemptinne et al., 2000). In my study, the alkaloids in *A. bipunctata* eggs may have caused at least some of the observed mortality in *C. septempunctata* larvae. Mortality was higher in larvae fed four eggs (trial 1) than in those fed only one egg (trial 2), suggesting that the number of eggs (and increased amount of alkaloids) fed to test larvae had some influence on larval mortality. Another potential cause of the high larval mortality observed during the first trial was the observation of multiple pathogens including microsporidia and bacteria. Although the bacteria observed are likely spore-forming *Bacillus* spp., bacterial spores were not observed in smear preparations of infected beetles. These bacteria were not present in the male and female parents collected from the field. Rather, they became prevalent during the trial. The origin and effect of these bacteria are not known.

There are few reports of bacterial pathogens in phytophagous coccinellids. Most studies on bacterial infections of Coccinellidae concern male-killing bacteria, including *Rickettsia*, Spiroplasma, *Wolbachia*, Flavobacteria, c-proteobacterium, and some agents that are yet to be identified. For example, *Rickettsiella stethorae* causes larval mortality in five *Stethorus* species (Riddick et al., 2009) and male-killing bacteria are able to infect several lady beetle species as well as other arthropod hosts.

The *Bacillus cereus* group is comprised of Gram-positive bacteria known for their pathogenic effects on a wide range of vertebrate and invertebrate animals. In this group is *Bacillus anthracis*, the causative agent of anthrax; *Bacillus cereus*, a bacterium commonly associated with food poisoning, and *Bacillus thuringiensis* (*Bt*) a bacterium developed as a microbial insecticide (Raymond et al., 2008). Ping et al. (2008) describe a WZ-9 strain of *Bacillus thuringiensis* that is harmful to the twenty-eight-spotted potato ladybird, *Henosepilachna vigintioctomaculata F*., larvae but not adults. The LC50 to second instars was 2.95 107 cells/ml after 72 h (Riddick et al., 2009). Co-infection of gypsy moth (*Lymantria dispar L*.) larvae with Bt and some strains of B. cereus may lead to synergistic interactions, including an increase in larval mortality (Saxena and Stotzky, 2000). Also, Bacillus subspecies produce toxins that are lethal to several lepidopteran, coleopteran, and some dipteran larvae (Saxena and Stotzky, 2000; Adang et al., 1985; Maagd et al., 2001).

4.2 Effects of Nosema adaliae on larval development and mortality

The results from my study suggest that *C. septempunctata* is an unfavourable host for *N. adaliae*. There was no significant difference in larval development between uninfected and *N. adaliae*- infected larvae and these results suggest that the pathogen has no effect on larval development.

In a previous study, *C. septempunctata* larvae fed *Tubulinosema hippodamiae*-infected *Hippodamia convergens* eggs took significantly longer to develop than larvae fed uninfected eggs, (18.7 and 17.7 days, respectively; Saito and Bjørnson, 2008). In another study, development of *C. septempunctata* larvae fed microsporidia-infected *H. convergens* eggs was significantly shorter than for larvae fed uninfected eggs (Saito

and Bjornson, 2006). In my study, larval development and mortality did not differ between control and treatment larvae for *C. septempunctata*. These results are expected because microsporidia are known to cause chronic sub-lethal effects that lower host fitness and often do not kill their hosts outright (Tanada and Kaya, 1993).

In my study, two control larvae became infected with microsporidian spores. The source of this contamination is unknown. Control and treatment larvae were maintained on different shelves within the same environmental chamber throughout the trials. Although these treatments were separated spatially within the chambers, and great care was taken to prevent contamination (control larvae were handled before treatment larvae, utensils were cleaned regularly in ethanol), it is possible for microsporidia to be inadvertently transmitted through air currents within the chambers or when handling. Regardless of how these test larvae became infected, the two infected individuals from the control were not included in the data analyses.

Several species of microsporidia have been reported in beneficial arthropods and they affect them in different ways. The microsporidian pathogen *Nosema granulosis* has a weak effect on the growth of the immature amphipod crustacean *Gammarus duebeni* Liljeborg, but has no significant effect on host mortality and fecundity in naturally and laboratory-infected individuals (Terry et al., 1998; Dunn and Rigaud, 1998; Ironside et al., 2003). In the European corn borer *Ostrinia nubilalis* Hübner, *N. pyrausta* causes chronic and nonlethal disease. Depending on the intensity of the infection and age of the host, *N. pyrausta* causes a decrease in egg production and adult longevity. The pathogen also reduces larval survival and delays larval development.

The microsporidium *N. locustae* infects several grasshopper and locust species, causing chronic disease connected with reduced feeding, development, and fecundity, in addition to increased mortality rates. Sublethal infections in locusts cause them to change from the gregarious form to a less damaging, individual stage. Fundamentally, *N. locustae* infects the fat body, which leads to a disruption of metabolism and energy storage. When infections are severe, the fat body is hypertrophied and filled with spores (Bjørnson and Oi, 2014). In the case of honey bees, infection by *Nosema* causes digestive disorders, shortened life spans, reduced population size of honeybee colonies, and decreased honey production and pollination (Chen et al., 2008).

4.3 Sex Ratio

In general, parasitic sex ratio defects are common among arthropods and probably affect sexual selection in several species. Parasitic sex-ratio distorters are a major selective force in the evolution of host mating behaviour and mate choice. Intracellular parasites increase their transmission capacity by manipulating host reproduction. Sex ratios may become more female dominant as a result of male killing or feminization of the host by the pathogen (Dunn et al., 2006). Some microsporidia alter the sex ratio of arthropods, resulting in female-biased progeny. An example is the female-biased sex ratios that result from microsporidia infection of the predatory mite, *Phytoseiulus persimilis* Athias-Henriot (Bjørnson and Keddie, 1999). However, the microsporidium *Tubulinosema hippodamiae* did not alter sex ratios of *Adalia bipunctata, Coccinella septempunctata*, or *Harmonia axyridis* when they were fed microsporidia-infected *Hippodamia convergens* eggs as larvae (Saito and Bjørnson, 2008). In my study, the sex ratio of emerged adults differed significantly, suggesting that *N. adaliae* has a

detrimental effect on the development of *C. septempunctata* males.

According to Terry et al. (1998), the microsporidium *Nosema granulosis* alters the sex ratio of the amphipod *Gammarus duebeni*. In this case, the pathogen changes males into functional females (Haine et al., 2007). Wittner and Weiss (1999) found the first evidence for a positive relationship between a microsporidian infection and host survival. Host survival was higher in the freshwater amphipod *Gammarus roeseli* offspring infected by *Nosema granulosis* when compared to those from uninfected mothers. *Nosema granulosis*-infected *G. roeseli* females reproduce earlier than do uninfected females, which may provide some sort of reproductive advantage for this species (Haine et al., 2004, 2007).

Sex ratio alterations arise from interactions between many factors including, sex determination, sex allocation, haplodiploidy, sex ratio distorting elements, or unusual life histories of parasitoid wasps. Other factors are responsible for sex ratio distortions in the Hymenoptera. Some microsporidian pathogens have the potential to suppress or eradicate an insect population or to reduce its productivity (Luck et al., 1999).

4.4 Vertical transmission of *Nosema adaliae*

Microsporidia have been reported from several lady beetle species and recent studies provide evidence that some of these pathogens are able to infect several hosts that are closely related (Bjørnson et al., 2011). Microsporidia may be transmitted either horizontally (from an infected to uninfected host through fecal or oral routes or vertically (from parent to offspring). Horizontal transmission is well documented for *Nosema ceranae* and *N. apis*, two pathogens that infect the midgut tissues of

honeybees (Didier et al., 2004). Microsporidia may also be transmitted horizontally when hosts are cannibalized, a common observation among lady beetles.

In a previous study, vertical transmission of *Tubulinosema hippodamiae* in *C. septempunctata* larvae was higher than what was observed in this study. Saito and Bjørnson (2008) detected microsporidia spores in 12.5% of the eggs of *C. septempunctata* that were produced on the first day of egg production and in more than 80% of the eggs that were produced seven days later. In my study, microsporidian spores were not detected until Day 8 and after that the proportion of eggs infected with spores increased until it reach 100%. This may be due to a difference in the pathogen: *T. hippodamiae* was the focus of the previous study but my study focused on *Nosema adaliae*. When a host ingests spores, the spore extrudes polar tube, which injects a sporoplasm (the infective stage) into the host cell cytoplasm of the midgut cells. The sporoplasm multiplies by merogony and the cell divides into two daughter cells or sporonts (by binary fission) or multiple cells (multiple fission). These cells then develop into mature spores (sporogony). During sporogony, a thick wall is formed around the spore, which provides resistance to unfavourable environmental conditions. When the spores increase in number and fill the host cell cytoplasm, the cell membrane ruptures to release the spores. Some of these spores re-infect the host but others are deposited in the feces or remain inside the carcasses of larvae or pupae that died of the disease. Most microsporidia undergo multiple rounds of merogony before they enter the sporogony phase, producing many spores. In *Nosema* sp., however, sporogony is weak, resulting in the production of two spores from each sporont (Vega and Kaya, 2012; Weiss and Becnel, 2014).

In the case of the parasitoid *Muscidifurax raptor* Girault & Sanders, all larvae and adults became infected after feeding on suspensions of *N. muscidifuracis* spores and larval mortality increased. In contrast, housefly (*Musca domestica* L.) larvae and adults did not become infected with *N. muscidifuracis* and there was no increase in mortality when compared with individuals from the control. Results also indicate that 25% of uninfected females and 17% of uninfected male adults become infected with *N. muscidifuracis* after feeding on infected *M. raptor* pupae. Infected *M. raptor* females produce less than 10% of the progeny that are produced by uninfected females. All of the offspring from infected females were infected with *N. muscidifuracis* but male parasitoids are unable to transfer the infection to their offspring (Geden et al., 1995).

4.4.1 Effects on Adult fecundity and longevity

In this study, the pathogen did not affect adult fecundity or longevity of *C. septempunctata.* These results were unexpected because microsporidia are known to cause chronic disease that results in lower host fitness, including low reproduction, growth or alterations of behaviour (Tanada and Kaya, 1993). Saito and Bjørnson (2008) did not observe differences in fecundity among *C. septempunctata* females fed uninfected and *T. hippodamiae*-infected *H. convergens* eggs. In my study, the fecundity of *C. septempunctata* from the control and treatment groups did not differ significantly.

Fecundity of *C. septempunctata* is dependent on the types of aphids used for food (food quality). Green peach aphids, *Myzus persicae*, used in this study had an effect on the number of eggs produced by *C. septempunctata*. When fed cotton aphids (*Aphis*

gossypii Glover) during a previous study, *C. septempunctata* produced 1660.5 eggs, but when fed cowpea aphids (*Aphis craccivora* Koch), *C. septempunctata* females produced only 1060.7 eggs (Kawauchi, 1985).

Results from another study show that uninfected and *T. hippodamiae*-infected *C. septempunctata* produce 2111 and 1900 eggs, respectively, when larvae were fed green peach aphid that was augmented with bird cherry-oat aphids (Saito and Bjørnson, 2008). In my trial, *C. septempunctata* laid a total of 2984 eggs (control) and 1290 eggs (treatment) when larvae were fed *Myzus persicae*.

The effects microsporidia on host fitness is likely pathogen and host specific (Haine et al., 2007). The results from a previous study (Saito and Bjørnson, 2008) suggest that introduced, invasive lady beetle species (*C. septempunctata* and *H. axyridis*) are somewhat resistant to the microsporidium *T. hippodamiae*. In this case, low spore counts suggest that *C. septempunctata* is less suitable for the reproductive success of the pathogen than are other native lady beetle species. Results from my study indicate that *C. septempunctata* may show some resistance to *N. adaliae*. Microsporidian spores were observed in the majority of smear preparations of individuals that were fed microsporidia-infected eggs; however, development of control and treatment larvae did not differ significantly. Although larval mortality was high for control and treatment larvae, mortality did not differ significantly.

Conclusion

This study was designed to investigate the effects of the microsporidian pathogen *Nosema adaliae* (from *Adalia bipunctata*) on larval development based on mortality, sex ratio, adult fecundity of *Coccinella septempunctata*. Microsporidia are known to

cause chronic disease that results in various negative effects on their hosts. In this study, significant differences in larval development, mortality, adult longevity and fecundity were not observed. However, the sex ratios between individuals in control and treatment groups differed significantly. *C. septempunctata* adults that were fed *N. adaliae*-infected eggs as larvae produced significantly more females offspring than males. Further studies are required to better understand the interactions between microsporidia and the lady beetles used for biological control in agriculture.

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