Horizontal Transmission of the Microsporidium, *Nosema adaliae*, from the two-spotted lady beetle, *Adalia bipunctata,* to the green lacewing, *Chrysoperla carnea*

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Abstract

The green lacewing, *Chrysoperla carnea* Stephens, and the two-spotted lady beetle, *Adalia bipunctata* L., are two natural enemies commonly used in biological control in North America. They are used in greenhouses and agriculture through augmentative release, and are mass-produced in commercial insectaries in Europe. Both have been found to host different species of microsporidia; however *Nosema adaliae* has been successfully identified and maintained within *A. bipunctata,* having a chronic effect on its host. Due to coexistence of the two insects, horizontal transmission of *N. adaliae* from *A. bipunctata* to *C. carnea* will provide knowledge of host specificity of the pathogen and lacewing susceptibility. The objective of this study is to determine if *N. adaliae* is successfully transmitted through oral consumption, if dose affects transmission, and if the pathogen has effects on *C. carnea* larval development. Three treatments of varying numbers of infected and non-infected *A. bipunctata* eggs were fed to *C. carnea* larvae, and development was observed over 30 days. Experimental trials were conducted under controlled environmental conditions. Test larvae were examined for microsporidian spores upon death or after the 30 days trials had concluded. The microsporidium was transmitted to two lacewing larvae that died early in their development suggesting acute effects of the pathogen. Low pathogen transmission suggests pathogen resistance and poor susceptibility of *C. carnea* to *N. adaliae*.

[April 18th, 2016]

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1. Introduction

In North America, both *Chrysoperla carnea* Stephens and *Adalia bipunctata* L. are commonly used for biological pest control of in agroecosystems, personal gardens and greenhouses. These two predatory species are mainly aphidophagous, feeding primarily on soft-bodied pests, and resorting to alternative food sources when necessary, including cannibalism. Both *C. carnea* and *A. bipunctata* are mass-produced in commercial insectaries for biological pest control, and their coexistence in agroecosystems provides an opportunity for insect pathogens to be transmitted from infected individuals to uninfected hosts within a given population through vertical and horizontal transmission. Such incidences may occur when infected individuals are unintentionally shipped to other regions for mass release. With world trade continuing to develop, and many commercial insectaries being in Europe, there is an increased likelihood for insect pathogens to be introduced in non-native and unintended territories (DeBach and Rosen, 1991). Better knowledge of the characterization and transmission of pathogenic species is essential to improving methods of biological control.

1.1 Biological and Chemical Pest Control

In recent years, agriculturalists have begun to incorporate more biological methods for controlling pest species on crops, and decreasing the use of pesticides and insecticides. Biological control encourages a more environmentally friendly approach than the use of chemical insecticides. Insecticides are relatively cheap compared to using biological control agents, and the confidence in the effectiveness of insecticides is high. In more recent years, biological control has gained more

attention with respect to the efficacy of using natural enemies and is becoming more economically feasible.

Chemical pest control began in the 1940s with the production of synthetic organic insecticides (DeBach and Rosen, 1991). In the U.S. Congressional Office of Technology Assessment, it was reported that the use of pesticides grew tremendously in the United States between 1950-1980, and doubled between 1964-1978 (Barbosa, 1998). Pesticides may be toxic, not only to target insects but also to non-target animals when chemically-treated plants are consumed, and also potentially through the consumption of poisoned insects. Pesticides may be effective at controlling pest populations; however, they are not a permanent solution. Pesticides must be applied to infested regions routinely. Furthermore, pesticides have been massively overused and misused resulting in environmental problems (Carson, 1962). It has been studied that even the smallest quantities (parts per million) of chemical pesticides, can have physiological effects on animals, and even be passed from mother to offspring. (Carson, 1962).

Dichlorodiphenyltrichloroethane (DDT) was one of the first chlorinated hydrocarbons used for pest control, having the capacity to act among a large range of pests and possessing long residual effects (DeBach and Rosen, 1991). Chlorinated hydrocarbons were soon replaced with organophosphorus and carbamate pesticides, which had shorter residual effects, yet were highly toxic to pests. The toxic effect of these chemicals also impacts humans, with 400,000 to 2 million pesticide poisonings occurring globally each year, with many resulting in death (DeBach and Rosen, 1991). Chemical insecticides also have the capacity of working

their way into non-target ecosystems through rain seeping from fields into nearby water systems, through wind causing the pesticides to blow to non-desired regions of land, and simply through the accidental consumption of plants treated with pesticides by humans and livestock. Thus, it is apparent that the use of chemical pesticides can have major negative effects to the environment and as a result, pesticides do not provide a sustainable method of pest control.

The excessive use of pesticides can also lead to pest resistance, which creates additional problems for pest control as the chemicals once used become less effective (Barbosa, 1998). Furthermore, the amount of labour and economic costs associated with chemical pest control is significant. The cost for pesticides amounts to over 3 billion dollars a year in the United States alone (DeBach and Rosen, 1991). Due to the ecological impact and detrimental effects associated with chemical pesticides, pest control has begun to shift to a more biological approach, with the use of insects and other organisms for biological pest control.

Contrary to chemical control, biological pest control is the control of pest species through the use of natural enemies that have a specific mode of action and are mass released into a specific habitat (Eilenberg et al., 2001). There are different strategies for implementing biological control: one is conservation, which is the use of the natural enemies that are native to a specific site and simply involves manipulation of the habitat to better enhance their survival (Kalia and Mudhar, 2011; Barbosa, 1998; Landis et al., 2000). Habitat manipulations can include building structures for enhancing survival, and offering additional food sources such as honeydew and more soft body insects for when pest concentrations are low.

Alongside conservation is the commonly used method of augmentation, which is when natural enemies native to a given site are mass reared and then mass-released at one time. Another strategy is classical control, which is when non-native natural enemies are imported from their country of origin and introduced to a new site where they did not exist previously. In this case, the pests have been introduced into a foreign area, usually by accident. The natural enemies for the given pest are then sought for in the region of pest origin and introduced into the foreign area as a means to control the host pest (Kalia and Mudhar, 2011).

Biological control is currently recognized as a viable alternative to pesticides in pest management, however using natural enemies over insecticides remains more expensive and difficult to maintain in large outdoor fields. This disadvantage makes biological control used less often than pesticides, which can easily and efficiently cover a field, and at lower costs. Across the world there is a drive to reduce pesticides and integrate a more biological approach to achieve adequate pest control. For example, in 1995, the United States Department of Agriculture called for the use of integrated pest control methods on 75% of all croplands by the year 2000 (Barbosa, 1998). As another example, Agriculture and Agri-Food Canada state that natural enemies control aphids quite efficiently; however, when climate is favourable, aphid populations can expand rapidly requiring the use of chemical control (2005). Before insecticides are used, Agriculture and Agri-Food Canada recommend to avoid spraying predators, and to take damage caused by the aphids into consideration, and assure that plant damage is minimized (2005). These recommendations effectively recommend an integrative approach where natural

enemies are used as primary pest control agents, and chemical use is restricted to those instances where pest populations are too abundant to control strictly through biological means.

Although predacious insects have been used effectively for biological pest control as early as 900 AD, farmers view biological control methods as ineffective and not easily implemented in agricultural crops (Barbosa, 1998). Critical reviews suggest that the augmentative release of natural enemies accompanied with the application of low-risk pesticides may provide the best opportunity for the implementation of biological control (Collier and Steenwyk, 2004). In order to overcome the challenges associated with augmentative release, such as migration of natural enemies out of the release area and the environmental disadvantages of chemical pest control, conservation biological control tactics must be implemented into more integrated pest management programs. Furthermore, efforts must be made to thoroughly understand the biology of the insects used, including their life history, reproduction, and host microorganisms such as symbiotic microbes and pathogens.

1.2 Two-spotted lady beetle: Adalia bipunctata

Adalia bipunctata belongs to the family Coccinellidae. This rather small (3.5-5.2 mm long) beetle has two color morphs. The non-melanic form has red elytra with two black spots, and the melanic form has black elytra with four red spots. The life cycle of *A. bipunctata* begins with the egg, which is often laid in clutches. Eggs hatch to become larvae, which go through four instars and pupate into adults. The adult emerges approximately 5 days later, at which point the sex of the adult can be

determined by observing their abdomen under a dissecting microscope. Both the male and female secrete pheromones, which attracts the opposite sex and initiates mating. Lady beetles mate multiple times to ensure fertilization, which is energetically expensive for females. Interestingly, females have the ability to store male sperm for up to a couple months. In order to reproduce, female lady beetles must consume an adequate diet of aphids, which provides sufficient energy and a protein rich diet that is a requirement for egg production. *Adalia bipunctata* consume a range of soft bodies insect and arthropod species, however; aphids are preferred as food.

Lady beetles have been commercially available for biological control since 1999 (De Clercq et al., 2005). Lady beetles are efficient at consuming a large quantity of aphids; however, they are likely to disperse when aphid populations are low and they are difficult to restrain within a given area following augmentative release. Because they consume large numbers of aphids, lady beetles are efficient at reducing aphid populations within outdoor areas when aphid populations are high, or in enclosed cropping systems (greenhouses).

Lady beetles are phototactic and move towards blue-green light, which helps them migrate towards aphids on host plants (Pervez, 2005). Some aphid species feed on one specific type of plant, however some species such as the green peach aphid, *Myzus persicae* Sulzer, feed on a wide range of host plants. Aphids that feed on plants with high glucosinolate concentrations, such as cabbage or horseradish, are more toxic to *A. bipunctata* than are aphids that feed on plants that are low in this secondary plant metabolite (Pervez, 2005). Interestingly, newly emerged *A.*

bipunctata larvae are attracted towards the chemicals released by plants that have been recently consumed by aphids. These chemicals stimulate the alarm pheromone in the larvae, cuing them to share in feeding. This social feeding in the early stages helps assure survival to the next instar (Pervez, 2005).

Lady beetles have been known to host a number of symbionts, including microsporidian pathogens (Steele and Bjornson, 2012). Microsporidia are spore-forming fungal pathogens that prolong larval development but have no effect on sex ratio, or adult fecundity and longevity (Steele and Bjornson, 2012). Due to the chronic effects of this pathogen, and its ability to be vertically and horizontally transmitted, it is possible for microsporidia to be transmitted to other susceptible natural enemies that share the same local environment.

1.3 Green Lacewing: Chrysoperla carnea

Chrysoperla carnea adults lay singular eggs that hang from a long stalk, and generally take 2-3 days to hatch after they are laid. Once the larvae hatch, they go through three instars, which take 8-12 days to complete. After sufficient time in the third instar, the larva secretes a silk substance and forms a cocoon and enters its metamorphic pupae stage. The adult emerges approximately 8 days later, at which point the sex of the adult can be determined by observing their abdomen under a dissecting microscope. Larvae are predacious, whereas adults feed solely on carbohydrate diets consisting of pollen. Since adults are winged and not predacious, they are likely to fly away from target areas. This is why the predacious larvae are released for biological control and not adults. Larvae consume soft-bodied pests,

notably aphids, and they are also highly cannibalistic when aphid populations are low.

Lacewings are known to host yeast symbionts; however, little research has focussed on how these yeasts are acquired and the potential benefits, if any, that they possess for the lacewing (Gibson and Hunter, 2005). Early studies suggest that yeast is acquired solely through the environment, and that yeasts present in the gut diverticulum supplement the adult lacewing with missing amino acids that are not acquired through their carbohydrate diet (Hagen et al., 1970). However, a more recent study was unable to replicate the former study, and researchers were unable to successfully isolate a yeast-free population of lacewings (Gibson and Hunter, 2004). The latter study suggests that yeast may be vertically transmitted, and this idea was supported by the observation of yeast on lacewing egg surfaces (Gibson and Hunter, 2005). In yet another study, yeasts were present in *C. rufilabris* larvae (Woolfolk and Inglis, 2004) but there is no definitive evidence of vertical transmission of these yeasts. Such symbionts have been reported from *C. carnea* larvae, and likewise no further studies have been conducted to determine the nutritional value of yeasts for this lacewing species (Gibson and Hunter, 2005).

C. carnea are known to host the microsporidian pathogen, *Nosema chrysoperlae*, which was found in lacewing larvae that had been obtained from a commercial insectary used as biological pest control agents (Bjornson et al., 2013). This demonstrates the susceptibility of the *C. carnea* to microsporidia and the potential for pathogen transmission among green lacewing larvae. Although *C.*

carnea is a suitable host for *N. chrysoperlae,* their susceptibility to other species of microsporidia is not yet known.

1.4 Microsporidia

Microsporidia are classified as obligate intracellular, spore forming fungi. These pathogens infect a range of invertebrate and vertebrate hosts (Dunn and Smith, 2001; Garcia, 2002). Microsporidia are eukaryotes and have a membrane bound nucleus, but their genomes are much smaller than those of other eukaryotes (Garcia, 2002). Microsporidia have no mitochondria, possess 70s ribosomes, and have very simple Golgi membranes. Evidence that microsporidia are related to fungi has been provided through gene sequencing and the presence of chitin in the microsporidian spore wall (Dunn and Smith, 2001; Garcia, 2002). Microsporidian spores are resistant to various environmental conditions and can remain infectious for years (Garcia, 2002). Spores are identified by their small size, thick walls, and a tightly coiled polar tube; the latter being a unique spore feature of all microsporidia (Keeling and McFadden, 1998). Microsporidia are very diverse and have many different characteristics with regards to their shape and size. Some microsporidia will vary in the thickness of their exospore and endospore walls, the number of nuclei they contain, and the structure of their polar tubes and the coiling that occurs within the spore (Dunn and Smith, 2001).

Microsporidia are transmitted both vertically (from parent to offspring) and horizontally (among cohorts). Vertical transmission in invertebrates is most commonly achieved through transovarial transmission. Vertical transmission is maternally linked because the cytoplasm of the zygote is acquired mainly from the

female rather than from the male sperm, which is significantly smaller and contributes a small amount to the overall mass of the zygote (Dunn and Smith, 2001).

Horizontal transmission occurs when the host ingests microsporidian spores, which can occur through the consumption of infected food. The microsporidia spore then germinates to infect the gut and eventually the pathogen spreads to other tissues (Dunn and Smith, 2001). The polar tube within the microsporidium can be everted quickly from the spore apex to penetrate the membrane of a nearby host cell. Upon penetration, the microsporidium injects the infectious material (sporoplasm) into the host cell where it develops and divides, eventually forming into mature spores (Keeling and McFadden, 1998).

1.5 Transmission of microsporidia

Previous studies have identified different microsporidian species in various lady beetles. One example is *Tubulinosema hippodamiae* from the convergent lady beetle, *Hippodamia convergens* Guerin-Meneville. This pathogen is vertically transmitted but it has also been successfully transmitted horizontally to several other lady beetle species, including *A. bipunctata*, under laboratory conditions (Saito and Bjornson, 2008). *A. bipunctata* is also known to host, *Nosema adaliae* (Steele and Bjornson, 2014). This newly identified microsporidium shares similar attributes to other microsporidia, such as *Nosema coccinellae,* with respect to spore size. Based on light microscopic observation of microsporidian spores, it is difficult to differentiate microsporidia within the same genus; however, internal spore structures differ for each species and these ultrastructural differences are

discernable when microsporidian spores are examined by means of transmission electron microscopy. Microsporidian that infect lady beetles tend to infect distinct tissues within the various lady beetles that they infect. This information can also be used to fully describe the pathology of these pathogens (Steele and Bjornson, 2014). Microsporidian species that belong to the genus *Nosema* develop in direct contact with host cell cytoplasm, and their spores have a distinctive spore wall (a thin endospore and thick exospore) and a distinctive polar vacuole (Sprague et al., 1992).

1.6 Transmission of microsporidia among more distantly-related species

Vertical and horizontal transmission of microsporidia within a particular host species is common, but some microsporidia are transmitted horizontally between closely-related host species. However, horizontal transmission of a particular microsporidium between insects that belong to different insect families has yet to be thoroughly investigated.

The microsporidium, *Nosema chrysoperlae*, has recently been characterized from *C. carnea* (Bjornson et al., 2013). Infected *C. carnea* show distinctive signs associated with infection. Some infected larvae turn black and die late in their development, and some infected adults emerge from their pupal cases with deformed wings that are thick and club-shaped (Bjornson et al., 2013). *Nosema furnacalis*, a pathogen of the same genus with similar structural characteristics to *N. chrysoperlae,* was not transmitted horizontally to *C. carnea* when larvae were fed solutions containing spores under laboratory conditions (Oien and Ragsdale, 1993).

This suggests that even though these two microsporidia are closely related, *C. carnea* is an unsuitable host for *N. furnacalis*.

Since some microsporidian species have been successfully transmitted to a variety of host species, it is reasonable to conclude that not all microsporidia are host specific and some may potentially infect a range of species as seen with *N. coccinellae* and *T. hippodaminae*. Since both lady beetles and lacewings host specific microsporidian pathogens of the genus *Nosema*, investigating the transmission of *N. adaliae* from *A. bipunctata* to *C. carnea* (a distantly-related host) is of interest from the perspective of host-pathogen interactions. Furthermore, these two natural enemies are widely used for biological pest control and are therefore likely to coexist in agroecosystems, especially if they are used simultaneously for pest control in a given area. This scenario would increase the likelihood for microsporidian pathogens to be transmitted from one natural enemy to another.

Microsporidia that infect lady beetles tend to cause chronic, delayed larval development. Although *N. adaliae* delays the development of *A. bipunctata* larvae, the pathogen has no effect on adult longevity, fecundity, or sex ratios (Steele and Bjornson, 2012).

The objective of this study is to examine the transmission of *N.adaliae* from the two-spotted lady beetle, *A. bipunctata* to the green lacewing, *C. carnea*. The effects of the microsporidium on lacewing longevity, fecundity and sex ratios will also be investigated. Our increased understanding of microsporidian transmission among insect natural enemies will help us better understand host-pathogen

interactions and the importance using pathogen-free natural enemies for biological pest control.

2. Materials and Methods

2.1 Caring of stock C. carnea and A. bipunctata

Chrysoperla carnea larvae were obtained from two shipments from BioBest Sustainable Crop Management, a supplier of beneficial arthropods located in Westerlo Belgium. Larvae were isolated individually in polyethylene Petri dishes (4.7cm diameter). Each dish lid had a 3.0cm diameter hole to which a fine mesh screen had been affixed. This allowed for air circulation and prevented the escape of test larvae. Prior to use, Petri dishes were washed, soaked in 10% bleach solution (10min), rinsed and left to air dry.

Chrysoperla carnea were reared in Petri dishes throughout their larval stage. They were fed green peach aphids daily until pupation. Water was provided through a moistened piece of cotton. Pupae remained untouched until they emerged. Once they emerged as adults, individuals were sexed, and then isolated into 120mL clear polyethylene cups. These cups were cleaned before use (as mentioned earlier) and a 2.2cm hole in the side of each cup was covered with fine mesh to permit air circulation.

To determine the sex of the *C. carnea*, soft tip forceps are used to hold the insects gently by their wings while exposing their abdomens under a dissecting microscope. The prominent, oval-shaped pad, located distally on the ventral surface, distinguishes females. In contrast, the abdomen of male lacewings lack this pad and their distal abdomen are pointed when compared to that of the females.

A. bipunctata were isolated and reared based on whether they were uninfected or infected with *Nosema adaliae*. *Adalia bipunctata* adults were reared in 120mL clear polyethylene cups (the same conditions that were provided for *C. carnea*). The cups were changed periodically, and each container was washed with soap, soaked in 10% bleach solution (10min), rinsed and left to air dry.

Both *C. carnea* and *A. bipunctata* were fed green peach aphids, artificial diet, and water which was provided through a moistened piece of cotton. The artificial diet consisted of equal parts of Lacewing and Ladybug Food (Planet Natural, MT) and pure unpasteurized honey. The artificial diet helps to keep lacewings in a given area by providing alternative food when pest populations are low, and also stimulates greater egg production. A small portion of the artificial diet was smeared on the inside of the cup. *Chrysoperla carnea* were only fed aphids during their larval stage. *Adalia bipunctata* were only fed aphids when required to lay eggs; all other times they were maintained on artificial diet and water.

2.2 Laboratory Conditions

The lady beetles and lacewings were kept in a chamber with a set temperature and light over a 24 hour time period (16:8 L:D; 25°C:20°C). The aphids were reared on nasturtium (*Tropaeolum nanum* L. Jewel Mized, Stokes Seeds, ON), a plant that grows quickly and has plenty of small green leaves for aphid populations to thrive.

2.3 Trial Set Up

To start the trial, eight *C. carnea* mating pairs were isolated in 120mL polyethylene cups, where they were supplied water and diet. The underside of each lid was lined with a piece of filter paper (55 mm, Whatman) for the eggs to be laid

on. Every two days, the mating pair was moved to a new cup and eggs were kept in the previous cup.

Uninfected and *N.* adaliae-infected *A. bipunctata* mating pairs were isolated in polyethylene cups and eggs were collected daily. To ensure the presence and absence of microsporidia in the infected and uninfected mating pairs, respectively, 4-6 eggs from each couple were smeared and stained for examination by light microscopy. The eggs used for trial were 24 hours old to prevent the possibility of the eggs hatching before the larvae had the opportunity to consume them. The eggs were also collected arbitrarily from different mating pairs.

Once the *C. carnea* larvae emerged from the eggs, they were fed aphids for 3 days to stimulate growth and development. At 72 hours old, 24 larvae were selected from various mating pairs were separated into individual Petri dishes and starved for 24 hours. Six larvae were allocated to each of the control and 3 treatment groups, each being from a different mating pair. In this way, a total of 24 larvae were separated daily for a period of 5 days allowing for a sample size of 30 larvae for each treatment and a total of 120 larvae for the trial. After the 24 hours lapsed, larvae were fed three uninfected or *N. adaliae*-infected *A. bipunctata* eggs, depending on the treatment group.

The control larvae were fed three uninfected eggs. Larvae in treatment 1 were fed two uninfected and one *N. adaliae*-infected egg; treatment 2 larvae were fed one uninfected and two *N. adaliae*-infected eggs; and treatment 3 larvae were fed three *N. adaliae*-infected eggs. The three eggs were placed on a small piece of filter paper

(5 mm diameter). The eggs and filter paper were placed into the centre of the Petri dish with one isolated *C. carnea* larva that had been previously starved for 24 h.

After 24 hours, the small piece of filter paper was removed. Larvae that failed to consume all three eggs were discarded. All other larvae were fed aphids and given water daily until pupation. The sex of each adult was determined following emergence. Smear preparations of all test individuals were made and these were stained with Giemsa and examined by light microscopy for microsporidian spores. The trial continued for 30 days, at which point any *C. carnea* that remained as larvae or pupa were smeared. When the trial was completed, the female parents of the mating pairs were smeared, as well as a six of the eggs from each mating pair. This trial was repeated with a second shipment of larvae.

2.4 Staining Procedure

Smear preparation of trial specimens were fixed in methanol (10 min), rinsed in tap water (10 min) and stained with 5% Giemsa (2 h). Smear preparations were then dehydrated in a series of solutions of ethanol :70% (3 min), 80% (3 min), 90% (3 min), 95% (3 min) and 100% (3 min), ending in xylene (10 min). Slides were then mounted with cover slips using Permount, and left to dry under a fume-hood overnight in an open cardboard jacket.

2.5 Light Microscope Analysis

The slides were examined under a compound light microscope (40 x magnification) for the presence or absence of microsporidian spores. Sample eggs of *A. bipunctata* used for the experiment were examined to confirm presence or absence of microsporidia within the clutches. Parents of *C. carnea* were smeared

and examined to confirm that they were microsporidia-free. Due to low transmission, simply the presence of microsporidia was noted, and not the variance in spore concentration.

3. Results

3.1 Transmission of microsporidia

Based on light microscopic analysis, only one specimen from treatment 1 was infected with microsporidia. This infected individual died in its larval stage after day 4 of the trial. When the trial was repeated (trial 2), only one specimen from treatment 3 was infected with microsporidia. This infected specimen died in its larval stage after day 3 of the trial. The infection status of test larvae from trial 1 and 2 has been combined and is presented in Table 1. Both specimens had a low count of spores, roughly 5-10 spores in each specimen. Parents of the two larvae that were positive for microsporidia tested negative. Transmission success was 1.92% in treatment 1 and 2.22% in treatment 3. Total transmission success for larvae that consumed any number of infected eggs was 1.37%.

Table 1: Combined total of lacewing specimens from trial 1 and trial 2 displaying count of *Chrysoperla carnea* with infected microsporidia

Treatment	Sample Size (n)	Microsporidia	
		Positive (+)	Negative (-)
Control	50		50
Treatment 1	52	1	51
Treatment 2	49		49
Treatment 3	45	1	44

3.2 Larval development

For analysis of development, combined data were analysed to determine significance in larval and pupal development. The Anderson-Darling normality test confirmed the data to be normally distributed. An ANOVA test was conducted to compare the average days spent in the larval stage within each treatment. ANOVA gave an F-value of 4.53 and corresponding P-value of 0.004 indicating a significant difference between at least one treatment group (Table 2). Figure 1 displaces the interval plot for the average number of days spent in larval stage with 95% confidence. A Tukey Pairwise Comparison determined where these significant differences were. This test showed that the control group was significantly different from treatment 1 and treatment 2, but not treatment 3. There were no significant differences between treatment groups (Table 3).

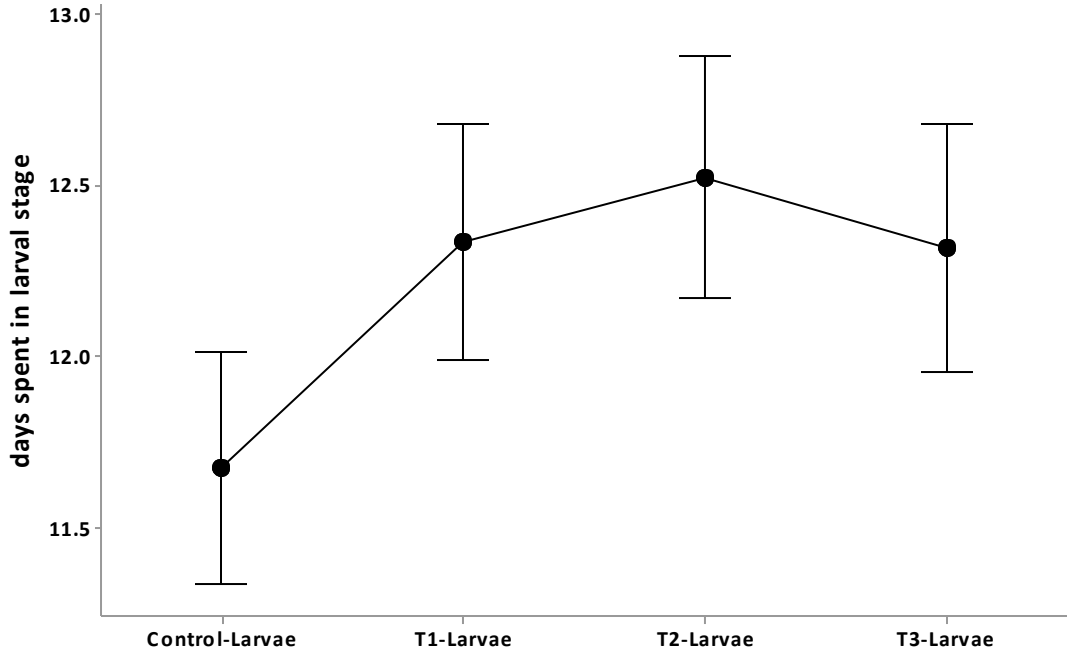
3.3 Pupal development

For analysis of pupal development, combined data from trial 1 and 2 were tested for normality using the Anderson-Darling normality test. An ANOVA test was conducted to compare the average days spent in the pupal stage within each

treatment. ANOVA gave an F-value of 3.50 and corresponding P-value of 0.017 indicating a significant difference in at least one treatment group (Table 2). Figure 2 displays the interval plot for the average number of days spent in the pupal stage with 95% confidence. A Tukey Pairwise Comparison determined the significant difference to be between treatment 3 and the control. No other pairings were significantly different from each other (Table 3).

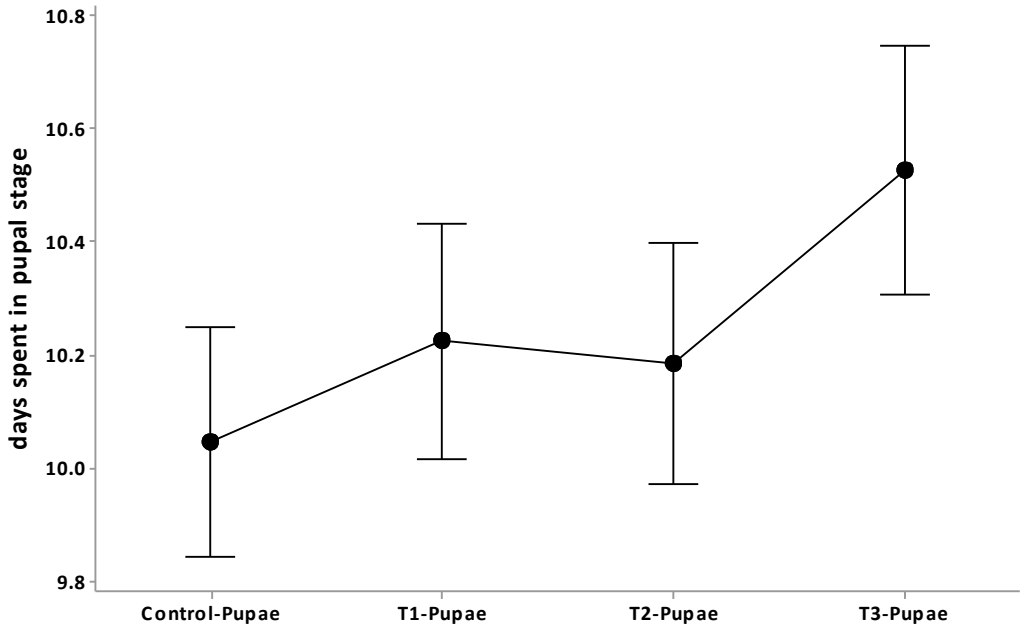
Table 2: ANOVA analysis of larval and pupal development based on average days specimens spent in both developmental stages between treatment groups. Sample size (*n*) includes only *Chysoperla carnea* that consumed all eggs in their assigned treatment.

	Larval Development				Pupal Development			
	n	Average days	St.Dev	P-Value	*n*	Average days	St.Dev	P-Value
Control	43	11.67	1.21	0.004	42	10.05	0.49	0.017
Treatment 1	42	12.33	1.05		40	10.23	0.48	
Treatment 2	40	12.53	1.18		38	10.18	0.61	
Treatment 3	38	12.32	1.07		37	11.67	1.21	



The pooled standard deviation was used to calculate the intervals.

Figure 1: Interval plot of displaying averages and standard deviations with 95% confidence interval of the number of days specimens spent in larval form within each treatment group.



The pooled standard deviation was used to calculate the intervals.

Figure 2: Interval plot of displaying averages and standard deviations with 95% confidence interval of the number of days specimens spent in pupal form within each treatment group.

Table 3: Tukey post-hoc test determined where the significant differences were located between treatments as indicated by letter groupings. Means that do not share the same letter are significantly different.

	Larval Development			Pupal Development		
	n	Average days	Grouping	*n*	Average days	Grouping
Control	43	11.67	B	42	10.05	B
Treatment 1	42	12.33	A	40	10.23	A B
Treatment 2	40	12.53	A	38	10.18	A B
Treatment 3	38	12.32	A B	37	11.67	A

4.0 Discussion

4.1 Transmission of microsporidia

Based on the results from this study, only 1.37% of the *C. carnea* were infected with microsporidia out of all the *C. carnea* that consumed at least one *N. adaliae*-infected egg. This low transmission percentage suggests that *C. carnea* have a high resistance to the microsporidium *N. adaliae*. Despite the low transmission, when the pathogen was successful, it caused death very early on in larval development. This differs from the chronic effects that *N. adaliae* has on *A. bipunctata* larvae, where development is prolonged but longevity is not hindered (Steele and Bjornson, 2012). Other *C. carnea* larvae that died early in the trial (n =7) tested negative for microsporidian spores, and with this observation in mind, the two infected *C. carnea* larvae could have died due to natural causes and not from infection by the microsporidium.

The parents of the two infected larvae were also examined. Microsporidia of the genus *Nosema* are difficult to differentiate from one another by light microscopy and if the parents were infected, it would difficult to conclude that the microsporidia was *N. adaliae* and not *N. chrysoperlae* or another species. However, the parents of the infected specimens were negative, thus providing evidence that the pathogen detected in the two infected larvae was *N. adaliae*.

These results broaden our current knowledge of microsporidia host specificity and host range. Although *N. adaliae* is similar in structure to *N. chrysoperlae* and other *Nosema* species, it is not surprising that transmission was low. Two other *Nosema* species have been examined to evaluate their success with

respect to horizontal transmission, but both were unsuccessful. *Nosema furnacalis* from the Asian corn borer, *Ostrinia furnacalis* Guenée, was successfully transmitted to the European corn borer, *Ostrinia nubilalis* Hübner, but not to *C. carnea* (Oien and Ragsdale, 1993). *Nosema pyrausta* was not successfully transmitted from *O. nubilalis* to *C. carnea* either (Sajap and Lewis, 1989). Therefore, despite similar mechanisms of infection and development within the host, the host range of these pathogens remains limited. Further transmission experiments with other microsporidian species that infect other arthropods used as natural enemies will further expand our knowledge of host specificity and pathogenic range within these hosts.

4.2 Larval development

Even though the vast majority of *C. carnea* larvae within the treatment groups remained uninfected, they took significantly longer to develop than those within the control group. The mean difference of approximately one day has implications for infected larvae, which remain vulnerable to cannibalism by cohort (and other lady beetle) larvae until pupation. Although the majority of *C. carnea* fed *N. adaliae*-infected eggs were uninfected, the delayed development observed for these larvae coincides with the prolonged development observed in *A. bipunctata* infected with this same microsporidium (Steele and Bjornson, 2012).

The alkaloids in *A. bipunctata* eggs are known to affect the development and/or survival of some lady beetle species (Hemptinne et al., 2000). In my study, all test larvae were fed 3 eggs each, regardless of treatment. This suggests that the alkaloids within *A. bipunctata* eggs are unlikely to be a contributing factor to the prolonged development that was observed. Lady beetles contain alkaloids as

chemical defense (Daloze et al., 1994); however, these alkaloids would be present within both the infected and uninfected eggs thereby ruling out the possibility that the alkaloids were responsible for the delayed development of *C. carnea* larvae. Concentration of alkaloids in the eggs was not determined; therefore it is possible that the eggs had varying concentrations of alkaloids. Since alkaloids are known to affect development, it is possible that differing alkaloid concentration may have caused the variance in development. It is also possible that the immune response of *C. carnea* has a negative impact on larval development as a tradeoff for resisting infection. Further investigation of *C. carnea* immune response and physiology are needed to substantiate this speculation.

4.3 Pupal development

Only treatment 3 larvae spent a significantly longer time in the pupal stage. This was the treatment with the highest dose of microsporidian-infected eggs, thus it is possible that the higher pathogen dose was responsible for prolonged pupation. However, none of the smears contained *N. adaliae*, therefore the higher microsporidium dose was not necessarily responsible for the prolonged development.

4.4 Implications for Biological Control

With respect to biological control, the results of this study provide some assurance to farmers and agriculturalists that this pathogenic microsporidium that normally infects *A. bipunctata* has a low risk of spreading from *A. bipunctata* to *C. carnea* when both used within a given area. Results show that only 1.37% of *C. carnea* will become infected when *N. adaliae* infected eggs have been consumed,

which is a small number with regards to the amount of *C. carnea* released in augmentative biological control. With this knowledge agriculturalists can be more secure with using both natural enemies at one given time.

Our knowledge of host specificity of microsporidia is not yet complete. Pathogen transmission could include other invasive or introduced lady beetle species, such as the seven-spotted lady beetle, *Coccinella septempunctata* L. and the multi-coloured Asian lady beetle, *Harmonia axyridis* (Pallas). As microsporidium species such as *Tubulinosema hippodamia* from *Hippodamia convergens* can be successfully transmitted to *A. bipunctata* and other lady beetle species, testing the transmission of this microspordium to *C. carnea* would provide more information regarding host specificity.

Having knowledge of pathogenic transmission is important for biological control because some pathogens may cause chronic disease for one species, but be lethal to another. Despite the low horizontal transmission that was observed during this study, *C. carnea* that fed on *N. adaliae*-infected *A. bipunctata* eggs took longer to develop than did those fed uninfected eggs; the reason for this is unclear. Further knowledge of host specificity will provide crucial information regarding the susceptibility of natural enemies to microsporidian pathogens when being mass reared in commercial insectaries or following release in biological control programs.

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