Effects of the Microsporidium *Nosema adaliae* from *Adalia bipunctata* L. on the

Multicoloured Asian Lady Beetle *Harmonia axyridis* Pallas.

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ABSTRACT

Originally imported for use as a biological control agent for pest insects, the multicoloured Asian lady beetle *Harmonia axyridis* has itself become a pest in many areas. While it is a very successful biological control, it has many non-target impacts such as displacement of native species of lady beetles and it can have adverse effects on human health. The geographic distribution of *H. axyridis* in Nova Scotia overlaps with the range of the native two-spotted lady beetle, *Adalia bipunctata*. This overlap provides the opportunity for the microsporidian pathogen *Nosema adaliae* to be horizontally transmitted to *H. axyridis*. In this study, *H. axyridis* larvae were allowed to consume a mixture of uninfected and infected *A. bipunctata* eggs. All *H. axyridis* larvae that consumed infected eggs were infected by the pathogen. Larval development was significantly prolonged for those larvae that consumed four infected eggs. These results suggest that *H. axyridis* has some resistance to the effects of *N. adaliae* since it requires the consumption of four infected eggs to significantly delay larval development.

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INTRODUCTION

Humans have long recognized the beneficial activity of predacious insects and parasitoids. The popularity of these organisms led to Emperor Francis I of Austria to command German naturalist, Vincent Kollär, to publish work on beneficial insects to aid the agricultural and forestry industries in 1837 (DeBach, 1965). Kollär praised the effectiveness of ground beetles and ichneumonid wasps for controlling harmful insects (primarily moths) (DeBach, 1965). Predacious coccinellids, commonly known as lady beetles, have also long been popular for pest control in agriculture. DeBach (1965) wrote that the benefits of lady beetles have been known to humans for centuries. The study of various beneficial insects in the 19th century to aid agricultural and forestry industries represents the beginning of modern biological control.

Biological Control

Biological control is the use of living organisms to control a variety of plant, animal, and fungal pests which are detrimental to human activities (Van Driesche & Abell, 2008). There are two major forms of biological control using captured (or bred) organisms: classical and augmentative (Van Driesche & Abell, 2008). Classical biological control is the importation of a natural enemy to control a foreign pest; normally the natural enemy is from the same region as the pest. Predacious lady beetles are very important in the history of classical biological control. The most successful example was the importation of the vedalia beetle, *Rodolia cardinalis* Mulsant from Australia (Gordon, 1985). In the late 19th century while the California citrus industry was

still young, it was nearly crippled by the cottony cushion scale *Icerya purchasi* Maskell, a pest species native to Australia. (Gordon, 1985). In response to this threat, Albert Koebele was sent to Australia in 1888 to locate potential natural enemies of the scale and send them back to America. Of the several species he imported, the vedalia beetle proved to be an immediate success and saved the industry in California. One citrus farmer, who had lost the previous year's crop to cottony cushion scale, reported that the use of the vedalia beetle cleared his entire 150 acre plot of scale insects (Caltagirone & Doutt, 1989). This success was the beginning of a wave of lady beetle introductions to North America as Koebele brought back more species in 1891 and 1892 (Gordon, 1985). Gordon (1985) wrote that roughly 26 species of imported lady beetles that were released for insect pest control had become established out of 179 introduced. This low success rate was due in part to the low rate of establishment. As a result, interest in predacious coccinellids waned in favor of hymenopteran parasitoids and synthetic insecticides.

Synthetic insecticides were originally seen as a major victory in the battle against pest insects of commercially important crops because of their effectiveness and low cost compared to other methods (Casida & Quistad, 1998). Over time, however, many adverse ecological effects began to emerge from the overuse of synthetic insecticides. In her book entitled "Silent Spring", Carson (1962) raised concerns about the effects of these chemicals on the environment and on human health. The concerns she raised led to stricter regulations and the formation of several government agencies (such as the Environmental Protection Agency) to oversee pesticide development and usage (Casida & Quistad, 1998). Growing public pressures, new regulations that made development of

pesticides more costly, and changing regulations caused a drop in the interest of synthetic pesticides (Casida & Quistad, 1998). This reduced use of chemical pesticides helped to fuel a renewed interest in the use of beneficial insects for biological pest control. The use of lady beetles increased dramatically in the 1960's and 70's (Gordon, 1985). However, the use of coccinellids no longer depended on importation from foreign countries with the goal of establishment. Instead, the practice of augmentative biological control rose in popularity. Augmentative biological control is the use of commercially reared insects applied on a routine basis for the purpose of reducing pest populations. Unlike classical control, augmentative biological control does not rely on the establishment of the control agent. Today, lady beetles are commonly used in augmentative biological control and many species are available for purchase from garden centers (Heimpel & Lundgren, 2000) (De Clercq et al., 2005).

Harmonia axyridis

One of the more successful imported beetles is the multicoloured Asian lady beetle, *Harmonia axyridis* Pallas. Imported as early as 1916 to California (Gordon, 1985), this beetle has since not only become established in North America, but has also outcompeted other native species. While its voracious appetite and hardiness makes it an excellent biological control agent, its ability to outcompete native species has led to it being recognized as an invasive species. Originally from Asia, *H. axyridis* has since spread to every continent except for Antarctica (Brown et al. 2011). *H. axyridis* was originally imported to North America in 1916 (Gordon, 1985) and was first reported as

established in 1988 (Chapin & Brou, 1991). Since then, it has been reported in all States/Provinces in North America except for Alaska, Wyoming, Saskatchewan, and Labrador.

H. axyridis is used to control aphids and other soft-bodied pests (Koch et al. 2006). Unfortunately, *H. axyridis* has a tendency to form large aggregations during the fall and winter and these aggregations may cause allergic reactions in humans (Nakazawa et al., 2007). These beetles also pose a risk to family pets and young children who may try and consume them. Stocks & Lindsey (2008) reported a case where a dog had consumed roughly a dozen *H. axyridis*. The dog then began to suffer from acute corrosion of the oral mucosa caused by chemical burns from alkaloids present in *H. axyridis* (Stocks & Lindsey, 2008).

H. axyridis is also considered to be an economic pest. There have been reports of aggregations feeding on produce such as apples, pears and grapes (Koch, 2003). In addition to feeding on produce, *H. axyridis* may negatively influence the processing of fruit. It has been suggested that *H. axyridis* infestation in vineyards taints the flavor of the resulting wine due to the alkaloids they produce (Koch, 2003).

An important non-target effect of the introduction of *H. axyridis* is its ability to out-compete native aphidophagous predators. Lady beetles are known to cannibalize their own eggs and larvae as well as prey upon the eggs and larvae of other lady beetle species (Agarwala, 1991). *H. axyridis* has been shown to benefit more (and suffer less) from these behaviours than native species in North America (Cottrell, 2005). In addition, *H. axyridis* also benefits more from predaceous intra-guild (a group of organisms that share

an ecological niche) interactions than do native species (Takizawa & Snyder, 2012).

Predation provides opportunities for pathogens to be transmitted from one beetle species

to another, one of these potential pathogens being the intracellular eukaryotic parasites

known as microsporidia.

Microsporidia

Microsporidia were first discovered in the 19th century when a strange disease

known as pébrine was infecting silkworms and threatening the southern European silk

industry (Keeling & Fast, 2002). Originally thought to be fungi, these organisms were

later classified into Phylum Microsporidia because the spores had a characteristic polar

filament, a unique structure used for initiating infection (Franzen, 2004). Molecular

evidence has since confirmed the remnants of mitochondrial genes in the microsporidian

genome that suggest the mitochondria was lost through reductive evolution (Keeling &

McFadden, 1998). Although microsporidia infect members of all five classes of

vertebrates and the majority of invertebrates (Mathis, 2000), microsporidia are more

commonly infect arthropods and fish (Keeling & Fast, 2002). Once thought to be

primitive parasites due to a lack of cellular structures such as mitochondria, more recent

studies suggest that microsporidia are highly evolved and specialized eukaryotes that

went through reductive evolution to better suit their parasitic lifestyle (Keeling & Fast,

2002).

Microsporidian life cycles are characterized by two major morphologies; an

infective spore stage and a reproductive, vegetative stage (Keeling and Fast, 2002).

Microsporidia are only able to survive host as environmentally resistant spores (Keeling

& Fast, 2002). These highly organized cells are comprised of a sporoplasm (the infective portion of the spore) that is surrounded by the spore wall consisting of an exospore and an endospore (Keeling & Fast, 2002). The sporoplasm of the spore contains one or more nuclei, depending on the species, and a number of unique structures that allow the spore to infect a host cell (Keeling & Fast, 2002), including the polar filament and polaroplast (Keeling & Fast, 2002).

When activated by environmental stimuli, the spore undergoes changes which cause a large build-up of osmotic pressure within the spore. This pressure leads to the discharge of the polar filament through the thinnest part of the spore's membrane, the anterior apex (Franzen, 2004). The polar filament is attached to the apex of the spore by the anchoring disc (Keeling & Fast, 2002). After the polar filament has been discharged and penetrates the host cell, the sporoplasm is forced through it into the host cell's cytoplasm (Keeling & Fast, 2002). Once inside the cell the microsporidia enters its vegetative stage during which it undergoes cell growth and reproduction (Keeling & Fast, 2002).

Microsporidia in lady beetles

There are a number of microsporidia known to infect coccinellid hosts causing a varying degree of negative effects (Riddick et al., 2009). Among these, at least five microsporidian species infect aphidophagous coccinellid species. Two microsporidia were discovered in *Hippodamia convergens* Guérin-Méneville: *Nosema hippodamiae* Lipa and Steinhaus (Lipa & Steinhaus, 1959), and *Tubulinosema hippodamiae* Bjørnson, Le, Saito and Wang (Bjørnson et al., 2011). Two other species of microsporidia infect the

seven-spotted lady beetle, *Coccinella septumpunctata* L.: *N. tracheophila* Cali and Briggs (Cali & Briggs, 1967), and *N. coccinellae* Lipa (Lipa, 1968). In addition to infecting *C. septumpunctata, N. coccinellae* was also found in other coccinellid species (Lipa et al., 1975). This shows that there is the potential for microsporidian pathogens to be transmitted between different coccinellid hosts. The fifth microsporidium found in aphidophagous coccinellids is *Nosema adaliae*. Found in a native Nova Scotian population of *A. bipunctata*, *N. adaliae* was shown to prolong larval development of its natural host (Steele & Bjørnson, 2012).

The host specificity of microsporidia in coccinellids appears to be fairly broad. Saito & Bjørnson (2006 & 2008) observed that an unidentified microsporidia (later identified as *T. hippodamiae*) from *H. convergens* was able to infect *A. bipunctata*, the two-banded lady beetle, *Coccinella trifasciata perplexa* Mulsant, *C. septumpunctata*, and *H. axyridis*. Not only was the pathogen able to infect the non-target host, it also caused a significant increase in larval development time (Saito & Bjørnson, 2006, 2008). The ability of a microsporidia to infect species from different geographical regions may also go in the opposite direction; native pathogens may be transferred to non-native species.

The distribution of *H. axyridis* into the native range of *A. bipunctata* in North America means that there is potential for *N. adaliae* (from *A. bipunctata*) to be horizontally transmitted to *H. axyridis* through intra-guild predation. This study seeks to determine if the microsporidia from *A. bipunctata* is successfully transmitted to *H. axyridis* and to determine the effects of the pathogen on this lady beetle. Also, the effect of consuming multiple infected eggs will be examined.

MATERIALS AND METHODS

Uninfected and microsporidia-infected *Adalia bipunctata* used in this study were obtained from laboratory-reared populations. Vertical transmission of *N. adaliae* in *A. bipunctata* is 100% (Steele & Bjornsøn, 2012); therefore, infection status was confirmed by smearing a sample of eggs from each parent group. Smears were then fixed in methanol, stained with 5% Giemsa solution (Sigma Diagnostics), and checked for the presence of microsporidian spores using light microscopy. *Harmonia axyridis* were collected from within 1 km of Saint Mary's University (923 Robie St, Halifax, NS B3H 3C3). Both *A. bipunctata* and *H. axyridis* were reared in clear 120 ml polyethylene cups (Canemco-Marivac Inc, QC) in separate environmental chambers under controlled conditions (16:8 L:D; 25°C:20°C). A 2-cm diameter hole was cut into the side of each cup to allow air circulation. The hole was covered with mesh screen (80 micron pores) to prevent beetles from escaping.

A cotton wick (Crosstex International, NY) placed in the cup was moistened daily to provide water for the beetles. All beetles were fed green peach aphids (*Myzus persicae* Sulzer) reared on nasturtium (*Tropaeolum minus* L., Dwarf Jewel Mixed; Stokes Seed Ltd., ON). Aphid colonies had been previously checked for microsporidia and were not infected. Plants and beetles were maintained in separate environmental chambers set to the same light-dark and temperature cycles as above (16:8 L:D; 25°C:20°C). Mating pairs from *H. axyridis*, *A. bipunctata* (uninfected), and *A, bipunctata* (infected) were stored in separate cups and eggs were collected daily. The first clutch of eggs from each

mating pair was smeared on a slide, fixed with methanol, stained with 5% Giemsa (Sigma Diagnostics), and examined using light microscopy to confirm infection status.

Test larvae were isolated in a clear polyethylene Petri dish (47mm diameter, Millipore Corp., MA) with a 2-cm hole in the top covered by mesh. Uninfected *H. axyridis* test larvae (24-48 h old) were divided into a control group and 3 treatment groups. Larvae from all groups were fed four *A. bipunctata* eggs: larvae that were fed 4 uninfected *Adalia bipunctata* eggs (Control); larvae fed 1 infected *A. bipunctata* egg and 3 uninfected *A. bipunctata* eggs (Treatment 1); 2 infected *A. bipunctata* eggs and 2 uninfected *A. bipunctata* eggs (Treatment 2); or 4 infected *A. bipunctata* eggs (Treatment 3). *Adalia bipunctata* eggs were presented on a small piece of moistened filter paper (6 mm diameter, Whatman International Ltd., UK) that was placed in the center of the dish on the first day of setup. Larvae were given two days to consume all 4 eggs and larvae were provided only water during this period. If the larvae consumed all 4 eggs they were then given an ample supply of aphids daily and allowed to mature. Larvae were observed daily to monitor development stage and/or mortality. Five larvae for each treatment group were set up each day for 6 days (30 individuals per treatment; n=120). This trial was repeated once following the same procedure.

Larvae that died after Day 4, pupae that failed to eclose, and adult beetles that eclosed were smeared, fixed with methanol, and stained with 5% buffered Giemsa solution (Sigma Diagnostics). These specimens were examined by light microscopy to determine infection status based on the presence or absence of microsporidian spores.

Data from larvae that did not eat all four eggs, and those that died prior to Day 4 were excluded from the data analyses.

Larval development data were tested for normality using a Shapiro-Wilk *W* test and were found to be non-parametric. Because of this, a Kruskal-Wallace test was used to determine significance among the four groups. A Dunn test was then performed to determine significance between groups.

Mortality data was collected for all larvae which survived for 48 hours after consuming all four *A. bipunctata* eggs. Mortality was converted into percentages and analyzed using a χ^2 test.

RESULTS

H. axyridis parents used to produce trial larvae did not contain microsporidian spores, nor did the egg clutches that were examined from these beetles. Uninfected *A. bipunctata* mating pairs that were used to produce uninfected eggs were also free from microsporidian spores. However, *A. bipunctata* mating pairs used to produce infected eggs and sample eggs collected from each of these parents contained microsporidian spores.

All control group larvae that were examined were free of microsporidian spores. Conversely, microsporidia were detected in all larvae smeared from treatment groups 1, 2, and 3 (fed 1, 2, and 4 infected eggs). There was a significant difference in larval development within the four groups ($H = 30.89$, $df = 3$, $P < 0.001$; Table 1). A significant delay in larval development time was observed between: the control group and treatment 3, treatment 1 and treatment 3, and treatment 2 and treatment 3 ($z = 2.638$; Table 1).

With respect to larval mortality, two data cells had expected counts less than 0 (Table 2); therefore, a χ^2 test could not be used to determine significance.

Table 1. Larval development time for control and three treatment groups

Treatment Group	N	Mean (Days)	Median	Avg Rank	Group
Control	29	19.03±1.70	19.00	37.2	A
Treatment 1[a]	26	19.65±1.96	19.00	46.2	A
Treatment 2[b]	31	20.26±1.39	21.00	57.9	A
Treatment 3[c]	23	21.74±1.21	22.00	83.5	B
Overall	109			55.0	

Kruskal-Wallace test (H=30.89;df=3;p<0.001);Dunn test z=2.638;Groups with different letters are significantly different.
[a] Larvae fed one *A. bipunctata* egg infected with *N. adaliae* and three uninfected *A. bipunctata* eggs
[b] Larvae fed two *A. bipunctata* eggs infected with *N. adaliae* and two uninfected *A. bipunctata* eggs
[c] Larvae fed four *A. bipunctata* eggs infected with *N. adaliae*

Table 2. Larval mortality for control and three treatment groups

Treatment Group	N	Mortality (%)
Control	29	0
Treatment 1[a]	29	10.35
Treatment 2[b]	31	0
Treatment 3[c]	27	14.82

[a] Larvae fed one *A. bipunctata* egg infected with *N. adaliae* and three uninfected *A. bipunctata* eggs
[b] Larvae fed two *A. bipunctata* eggs infected with *N. adaliae* and two uninfected *A. bipunctata* eggs
[c] Larvae fed four *A. bipunctata* eggs infected with *N. adaliae*

DISCUSSION

All *H. axyridis* larvae that consumed at least one infected *A. bipunctata* egg became infected with microsporidian spores. Those larvae that did not consume four *N. adaliae*-infected eggs showed no significant difference in larval development time (Table 1) even though they were infected with the pathogen. Larval development was delayed by about 2 days for those individuals that consumed four eggs at the beginning of the trial. This suggests that although *N. adaliae* is able to infect *H. axyridis,* the latter is not an ideal host for the pathogen and a larger initial dose of the pathogen is needed to incur observable, negative effects.

Horizontal transmission of microsporidia in coccinellids has been previously investigated with *N. tracheophila*, *T. hippodamiae*, and *N. adaliae*. In the case of *N. tracheophila*, the pathogen was transmitted from one natural host to another, showing that it could be transmitted horizontally through the consumption of infected eggs (Cali & Briggs, 1967). *Tubulinosema hippodamiae*, was successfully transmitted horizontally to its native host (*H. convergens*) as well as four other coccinellid species (*A. bipunctata*, *C. septumpunctata*, *Coccinella trifasciata perplexa* and *H. axyridis*) with high transmission rates (Saito & Bjørnson, 2006, 2008). Each of these hosts are distributed in Nova Scotia (Saito & Bjørnson, 2006, 2008). Prior to this experiment, *N. adaliae* had only been experimentally transmitted between *A. bipunctata* through egg predation (Steele & Bjørnson, 2012). *Nosema adaliae* transmission to *H. axyridis* was 100%, which is similar to the high transmission rate to its natural host *A. bipunctata* (Steele & Bjørnson, 2012). My results in combination with previous studies support the idea that microsporidia

which infect coccinellids have a fairly broad host range and are able to infect hosts from distant geographical regions.

Larval development time was significantly delayed only when *H. axyridis* larvae consumed four *A. bipunctata* eggs infected with *N. adaliae* (Table 1). This differs from the effects of the pathogen on its native host (*A. bipunctata*). Steele & Bjørnson (2012) found a significant difference in larval development time between *A. bipunctata* fed uninfected eggs and those fed a single, infected *A. bipunctata* egg and one uninfected *H. convergens* egg. The resistance of *H. axyridis* to the effects of *N. adaliae* differs from the effects of microsporidia on other lady beetles. Saito & Bjørnson (2006, 2008) report a significant delay in larval development time for *H. axyridis* larvae fed only one *H. convergens* egg that was infected with *T. hippodamiae*.

The difference in the number of *N. adaliae*-infected eggs needed to induce a significant delay in larval development may confer an advantage to *H. axyridis* over *A. bipunctata*. Because *H. axyridis* seems to show some resistance to the effects of the pathogen, it is possible that the ability of *H. axyridis* to consume a few *N. adaliae*-infected eggs plays a role in the success of *H. axyridis* in Nova Scotia where the population numbers of this beetle have been increasing (Cormier et al., 2000). The results in this trial were obtained under controlled conditions and different results may be observed in nature where a range of temperature fluctuations and other environmental factors are expected.

A case which highlights differences between laboratory results and natural settings occurs in lepidopteran species and their microsporidian pathogens. The gypsy

moth, *Lymantria dyspar* L., becomes infected with several microsporidian pathogens in Bulgaria (where gypsy moth is a native species) but not in North America where it is an introduced species (Solter & Maddox, 1998)(Solter et al., 2000). Furthermore, Solter & Maddox (1998) were able to infect *L. dyspar* with native microsporidia. The use of eggs to transmit the pathogen likely mimics what happens in the field. Coccinellids are known to consume other species' (and their own) eggs as well as engage in intra-guild predation (Agarwala, 1991). This provides reasonable evidence that *H. axyridis* larvae may come into contact with *A. bipunctata* eggs or young larvae and consume them.

Larval mortality data was not able to be analyzed because of statistical limitations. A higher sample size would likely produce results which could be analyzed for significance. *Nosema adaliae* does not incur significant larval mortality in its natural host *A. bipunctata* (Steele & Bjønson, 2012). Because *H. axyridis* shows some resistance to the pathogen when compared to *A. bipunctata*, it is unlikely that *N. adaliae* would produce a significant mortality for larvae that consume one infected egg. Since the number of eggs needed to initiate pathogenic effects on *H. axyridis* was not tested by Steele & Bjørnson (2012), it is difficult to predict the effects of consuming multiple infected eggs on larval mortality.

CONCLUSION

N. adaliae was able to infect *H. axyridis* with 100% horizontal transmission through egg consumption. Although all larvae contained the pathogen, only those that consumed four *N. adaliae*-infected eggs (treatment 3) showed significantly delayed larval

development (Table 1). Treatment 3 also had significantly delayed larval development time compared to the other two treatment groups (Table 1). This differs from the effects of *N. adaliae* in its natural host *A. bipunctata*, which showed a significant difference in larval development time after consuming one infected egg (Steele & Bjørnson, 2012). This may provide *H. axyridis* a competitive advantage in the field due to innate resistance to the pathogen.

     H. axyridis is a very successful invader which has become established in most of Canada and the United States (Koch et al., 2006). The range of *H. axyridis* overlaps with that of *A. bipunctata* in Eastern Canada and provides the potential for pathogen transmission. The microsporidian pathogen *N. adaliae* was found in *A. bipunctata* collected from Halifax, Nova Scotia (Steele & Bjørnson, 2012) and represents one of the native pathogens that could be transmitted to invasive species.

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