Effects of a microsporidium pathogen, *Nosema adaliae*, on the general predator Chinese Praying Mantis, *Tenodera sinensis*

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Abstract

Microsporidia are unicellular eukaryotic parasites that cause chronic diseases in invertebrates, as well as some other species in the animal kingdom, such as fishes, crustaceans and even humans. Microsporidia are known to cause a variety of effects to their hosts, such as increase in development time, increase in mortality, changes in feeding amounts, and increased likelihood of experiencing developmental abnormalities. *Nosema adaliae* is known to cause chronic disease to its natural host, the two-spotted lady beetle (*Adalia bipunctata* Linnaeus). *N. adaliae* may infect other lady beetles used as biological control agents. The focus of this study is to determine whether *N. adaliae* can be transmitted horizontally from *A. bipunctata* to the Chinese praying mantis (*Tenodera sinensis* Saussure) through direct feeding of infected beetle larvae. Horizontal pathogen transmission may occur as a result of intraguild predation, when these two biological control agents are used together for pest control. Three treatment groups were established from a population of *T. sinensis*, and each was provided with two second-instar *A. bipunctata* larvae: those from the Control group were provided two microsporidia-free *A. bipunctata larvae*, individuals from Treatment 1 were provided one uninfected and one microsporidia-infected *A. bipunctata larva*, and Treatment 3 individuals were provided two microsporidia-infected *A. bipunctata* larvae. Once these were eaten, *T. sinensis* nymphs were provided a diet of fruit flies (*Drosophila hydei*) and they were reared until they died. *N. adaliae* did not infect *T. sinensis* when infected beetle larvae were eaten. Mortality and longevity of *T. sinensis* did not differ significantly between groups. Failure of molt was significantly higher in Control individuals when compared to those of Treatment 1 or Treatment 2. Individuals of the Control group consumed significantly fewer fruit flies than did those from Treatment 1 or 2.

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Introduction

1.0 Biological Control

Biological pest control defines the practice of regulation and control of pests through the use of other living organisms, such as predators, parasites, nematodes or pathogens (Flint & Dreistadt, 1998). The objective of biological control is not to eradicate pests from the environment, but to control the pest population size by lowering it to a point where it will not cause noticeable harm (Mahr et al., 2008). The term 'biological control' was first introduced by H. S. Smith in 1919 and it indicated the use of both biotic and/or abiotic control factors, such as predatory insects and/or pesticides. In 1964, P. DeBach further defined "biological control" in order to better differentiate it from "natural control" (Johnson, 2000). Biological control was defined as a human induced control of pest populations through the manipulation of natural predators or parasites. On the other hand, natural control was defined as the fluctuation of a population size around an equilibrium point depending on the biotic and/or abiotic factors of the environment (Doutt, 1964). Robert van den Bosch, professor of entomology at the University of California, further defined natural control as "*control that occurs without man's intervention*" and applied biological control as the "*manipulation of natural enemies by man to control pests*" (van den Bosch et al., 1982). These definitions are currently in use by the scientific community.

Applied biological control is further differentiated into three categories: classical (or importation), augmentation, and conservation (Johnson, 2000). Classical biological control is practiced when an exotic pest is introduced into a new ecosystem. The accidental introduction of a foreign pest in a new area could cause great damage, especially when its natural predator is not present to keep the pest population size under control. To counteract the invasive species, the correct natural enemy (a predator or parasite able to decrease the population size of the pest species) would need to be found in its place of origin and released into the new area (Mahr et al., 2008). This practice often requires travelling to the country of origin of the pest and locating its natural enemy, to then release it in the new area where it did not exist previously (Huffaker & Dahlsten, 1999). For classical biological control to be successful, the correct taxonomic identification of the pest species is critical. A mistake in the identification of a pest may result in the introduction of an incorrect natural enemy, which may provide inadequate control of the pest or cause even further harm to the ecosystem. For this reason, natural enemies are quarantined and tested before release to examine their efficiency, as well as to assess any possible repercussions on the environment (Berryman, 1999). This technique usually requires the recruitment and potential release of a considerably large population of exotic natural predators, with the end goal of suppressing the pest population (Mahr et al., 2008). An example of classical biological control is the introduction of the multi-coloured Asian lady beetle, *Harmonia axyridis* Pallas, into North America. This lady beetle species is native of Eastern Asia. It was

introduced to North America to counteract the increasing population size of the aphid, *Aphis spiraecola* Patch, also native of Asia (Brown & Miller, 1998). Ironically, the Asian lady beetle was not properly vetted as a classical biological control agent, becoming an aggressive species in North America where it is well known to outcompete other lady beetle species (Roy et al., 2006).

Augmentation biological control involves the repeated introduction of a natural enemy into an environment where the natural enemy is already found. The objective of this technique is to increase the population size of the indigenous natural enemy species with the end goal to reduce, or manage, the population size of the pest (Mahr et al., 2008). Augmentation biological control can take place through either inundative or inoculative releases of natural enemies. Inundative releases result in the tentative control of a given pest only through the number of natural enemies released; therefore, it often requires numerous releases over time. Inoculative releases occur when the agents released are expected to produce viable progeny that build a population size large enough to provide control for the pest (Elzen & King, 1999). A general example of augmentation biological control is the periodic releases of *Aphytis melinus* DeBach, a parasitic wasp of the California red scale, *Aonidiella aurantii* Maskell. This parasitoid species is periodically released in California to protect numerous citrus cultivations of the region (Hoy, 2008).

Conservation biological control involves cultural practices that improve the survivability and efficiency of the natural enemies that are already present in the local

environment. Conservation biological control is used to increase the activity and/or density of such natural enemies (Ehler, 1998). A common technique involves the cultivation of specific pollinating plants within a monoculture crop to provide an alternate food source for those natural enemies that benefit from the intake of pollen for additional nutrients. Pollen provides energy that increases activity and reproduction, and it is known to be beneficial for many biological control agents, including the larval stages of some lady beetle species (McCravy, 2008). Another conservation technique includes the reduced or controlled use of pesticides. The chemicals contained in these products are able to harm both the pest and the natural enemy (Michaud, 2002). Conservation biological control is thought to be the safest option for the environment because it encourages the population growth of natural pest control agents and does not require the introduction of a foreign species in the ecosystem, which may damage the equilibrium of the habitat (Ehler, 1998).

Biological control is often referred to as a science made by trial and error, which has more often resulted in new problems arising due to newly introduced species. Only through research are we able to better understand the risks that are involved (Hawkins et al., 1993). Biological control is based on the correct taxonomic knowledge of pests, and therefore of their natural enemies; the correct use of beneficial organisms is critical for this practice to work effectively. For example, the incorrect identification of a pest could lead to the introduction of a beneficial pathogen that causes no harm to the target pest, but it may affect other organisms in a given area (Berryman, 1999).

Biological control can be traced back almost 2000 years to China, when insects were used to protect crops as early as 200 AD. In the book entitled 'Nanfang Caomu Zhuang' (Plants of the Southern Regions), the Chinese botanist Ji Han describes the use of 'yellow fear ants' (*Oecophylla smaragdina* Fabricius) for the purpose of controlling insect pests in 304 AD. These ants were sold for the protection of citrus fruits in the Canton region in China, and their use continues to this day (as cited by Temple & Needham, 1998).

Over the past 150 years, the use of biological control agents has exponentially increased, opening the field to a variety of research and industries profiting from the rearing and selling of biological control agents to the public (Hussey, 1985). In 2016, the world-wide biological control market was valued USD 2527.15 million, with a compound annual growth rate of 13.46% between 2017 and 2022, bringing the market value to USD 5245.15 million by the end of 2022 (Mordar Intelligence, 2017).

Although biological control is a practice that can be executed through different means and organisms, such as predators, parasites, nematodes or pathogens, this paper will focus on the generalist predator, the Chinese mantis (*Tenodera sinensis* Saussure) of the Order Mantodea.

2.0 Predatory Insects

Biological control agents may involve natural enemies from a variety of taxonomical groups, ranging from small insects, such as parasitic wasps of the Genus *Trichogramma*, to larger mammals, such as the Brazilian free-tailed bat, *Tadarida brasiliensis* I. Geoffroy (Flint & Dreistadt, 1998; Kunz et al., 2012). Some of the most commonly used biological control agents are predatory insects. These organisms are usually effective due to their large size (compared to the pest) and their ability to feed on numerous prey during their lifetime. Predatory insects can be classified under two broad groups: generalist and specialist, which differ based on prey specificity (Mahr et al., 2008). When compared to parasitoid insects, which are usually specific to one or a few species of prey, most predatory insects are classified as general predators due to the large variety of prey in their diet (Snyder & Ives, 2003). Generalist predators tend to be polyphagous, and often feed on different prey that belong to different taxonomical groups, even outside Class Insecta (Mortillaro, 2017). Some of the most commonly used general predators belong to Family Coccinellidae, also known as ladybirds, ladybugs or lady beetles. Predators that tend to feed on a variety of prey are effective for pest control in annual crops. This is because pests of annual crops are present only during specific times of the year, but generalist predators are able to survive until the following year by feeding on alternative prey (Symondson et al., 2002). Although generalist predators are more effective for controlling pest populations, they are slower to reduce the pest population than are specialist predators. This is due to the longer life cycle of

generalist predators when compared to the pests. For example, *T. sinensis* has a life cycle of 1 year, which is extremely long compared to most aphid species (Superfamily Aphidoidea) which have a generation time of a week and serve as prey (Mahr et al., 2008; Dixon, 2012). However, *T.sinensis* is not limited to a diet of aphids alone and it is used as natural enemy for a wide variety of pests (Mahr et al., 2008).

Specialist predators are biological control agents that feed strictly on one or a few species of prey. Some of the most commonly used specialist predators are mealybug ladybirds (*Cryptolaemus montrouzieri* Mulsant), which have a diet almost exclusively limited to scale insects. These predators usually have a shorter generation time compared to generalist predators, allowing them to be extremely effective in providing a quick reduction of the population size of a pest. On the other hand, the use of specialist predators for biological control has several disadvantages when compared to generalist predators. Specialist predators are unable to establish a self-sustainable population in annual crop cultivations due to the sudden disappearance of the pest at a certain time of the year. Furthermore, specialist predators are unable to prevent sudden outbreaks of pest populations since their population size is positively correlated to the population size of the pest (Symondson et al., 2002).

It is common to use more than one biological control agent to reduce the population size of any given pest. Between 1960 and 2001, significant decreases in pest populations in most cases were attributed to the use of an 'assemblage of generalist predators' or when 'natural enemies and generalist predators' were used. Even though

this procedure could potentially create great benefits and further reduce the population size of the pest, it is also possible to achieve an opposite result due to intraguild predation (Symondson et al., 2002). This happens when introduced general predator(s) feed on (or are fed upon by) another predator(s), reducing one or more populations of biological control agents, and diverting predation from the pest to another predator, therefore allowing the pest's population to increase. Intraguild predation may involve native or introduced predators. For example, in the Imperial Valley of California the release of the lady beetle *Delphastus pusillus* LeConte for whitefly control was ineffective due to intraguild predation by native general predators, including some heteropteran species (*Orius* and *Geocoris* spp), lacewings, lady beetles, spiders, and ants (Heinz et al., 1999). In this study, we consider the possibility of intraguild predation between two predators and we apply the concept to the transmission of a microsporidian pathogen from the two-spotted lady beetle, *Adalia bipunctata* Linnaeus, to the Chinese praying mantis, *T. sinensis*.

3.0 Mantodea (Burmeister, 1838): Taxonomy

The general term 'praying mantids' refers to any species that belongs to Order Mantodea. These insects are included in the group of orthopteroids due to the plesiomorphic features (homologous traits) shared with the members of the other orders in this group (Roger, 1999). The name of this group is derived from a previous

taxonomical classification that historically included praying mantids and many other species belonging to Order Orthoptera, which now includes all species of grasshoppers, crickets and locusts. Members of the orthopteroid orders are terrestrial insects that still show numerous ancestral traits, such as the presence of cerci, hemimetabolous metamorphosis, a large number of Malpighian tubules, and the presence of a large anal lobe on the hindwing (Marshall, 2006). There are more than 2,300 identified species of praying mantids, all of which are predaceous (Evans, 2007). The largest family is Mantidae, which includes the *T. sinensis*, the subject of this study.

3.1 Mantodea: Morphology and Function

Praying mantids have a large abdomen, composed of 10 tergites (dorsal plates) and seven or nine sternites (ventral plates), depending on sex: females possess seven and males possess nine. Females usually have a larger abdomen, which makes it easier to distinguish the sexes. In both sexes the abdomen ends with a pair of cerci; appendages used as primitive sensory receptors, which are a shared trait with other insects of the orthopteroid group. The abdomen is usually covered by two pairs of wings, which lay folded across the dorsal body surface. The ability to fold their wings places mantids in Infraclass Neoptera along with most winged insects. Some species of Mantodea are apterous (lack wings), and are defined as 'secondarily wingless', meaning that they have evolved from winged ancestors but have lost them in their evolution (Roger, 1999). There is much variation among the winged species; some are defined as brachypterous (short-winged), such as *Eremiaphila braueri* Krauss (Edmunds & Bunner,

1999) while other species are macropterous, or long-winged mantid, such as the previously mentioned *T. sinensis*. The variation in wings can also be due to sexual dimorphism. For example, males of the Tanzanian ground mantis (*Tarachodes afzelii* Stal) have long wings which allow it to fly, whereas females have vestigial wings and are flightless (Edmunds & Bunner, 1999). Flight involves direct flight muscles, which is a primitive feature whereby the flight muscles are directly attached to the wings. Other species in this order also possess indirect flight muscles. This alternative flight system is usually present in 'more evolved' insects, and involves rapid deformation of the insect's thorax, which allows the flapping motion of the wings (Hurd, 2009).

The hindwings are delicate membrane-like structures used for flight. They are often colourless and not visible when folded under the forewings (Roger, 1999). The forewings (also called tegmina) have aerodynamic features but they are not used in the flight itself; they are kept open while the hindwings are used for the flapping motion (Hurd, 2009). In the Mantodea, the tegmina are often green or brown and used for camouflage. Some species have adaptations to the tegmina that allow aposematic displays (warning coloration) when threatened and are often used for deimatic display (sudden display) to startle potential predators. In some cases, pigmentation patterns on the underside of the tegmina resemble two large eyes. The tegmina remain covered while the wings are folded, leaving the mantid camouflaged until threatened (Wootton, 2009).

The elongated prothorax allows the mantid to rotate its head almost 180 degrees (unique to the insect world), achieving a field of view of greater than 300 degrees when hunting (Evans, 2007). Two raptorial forelegs are attached to the prothorax. These are used for walking, but their main function is to grasp and hold onto prey. The raptorial legs are the most obvious characteristic of these insects; they are held in a position which resembles a person praying, from which the name 'praying mantids' arose (Marshall, 2006). On the femur and tibia of the forelegs, the mantid possesses a row of sharp spines, which allows the insect to lock the prey in place while feeding on it. The mesothorax allows the attachment of two middle legs that project forward and a pair of thickened tegmina in winged species. The metathorax attaches the hind legs as well as the hind wings, both used in locomotion.

Mantids have a triangular head with filiform antennae and five eyes: two compound eyes and three ocelli (Evans, 2007). The compound eyes are extremely large and well evolved. They are found on the sides of the head and allow mantids to see movement of potential prey from up to 60 feet away (Loomis & Stone, 2007). The ocelli are small and found on the upper centre of the head. These are used to perceive light intensity and movement (Evans, 2007).

Almost all praying mantids are ambush predators. They camouflage themselves while they wait for their prey to get closer, then strike and capture it with their raptorial forelegs. Once their prey has been captured, the praying mantid starts feeding on the appendages or the head, preventing the prey from escaping. The prey is chewed by

mandibulate hypognathous (lower jaw longer than upper) mouthparts, which can rapidly cut through the chitin-rich exoskeleton of other insects.

3.2 Mantodea: Development and Reproduction

Mantids are hemimetabolous, undergoing incomplete metamorphosis. This type of development is characterised by a life cycle with three stages: egg, nymph, and imago (adult). A pupal phase is notably lacking. The female mantid can lay 10 to 400 eggs, depending on the species and environmental factors. Eggs are laid in a case (ootheca), which protects them from low temperatures and environmental factors, such as desiccation or rain, until the following season (Elzinga, 1997). Although most species reproduce sexually, some species reproduce through parthenogenesis, whereby female individuals develop from oocytes without fertilization (Hurd, 1999).

In the spring, up to 400 juvenile mantids hatch from the oothecae and rapidly undergo their first molt. The nymphs resemble adults, but lack wings. Nymphs hunt small prey such as aphids and fruit flies, and they molt between 5 and 10 times depending on the species (Hurd, 1999). Adult mantids can live anywhere from 4 weeks to 6 months and usually die shortly after reproduction.

Females are usually larger than males and can lay several oothecae before dying (Hurd, 1999). Praying mantids are widely known for their peculiar behaviour of sexual cannibalism, which is exhibited in roughly 90% of all praying mantid species. This

behaviour involves predation of the male by the female before, during, or after copulation (Wilder et al., 2009).

Although a single oothecae may contain up to 400 eggs, only a small percentage of the offspring reach adulthood. Hurd and Eisenberg (1984) studied mortality, development, and dispersal of *T. sinensis*. Their results showed a juvenile mortality rate around 93%, leaving only a small percentage of mantids to reach adulthood. Part of their study included tests with a density of mantids significantly higher than in nature. This was done to favour interactions between individuals and encourage cannibalism. Even though cannibalistic behaviours between same instar mantids was recorded, it was not a significant cause of mortality, involving only 2% of deaths. Many other experiments indicate the presence of cannibalism between mantids, and results show that it is correlated to prey availability. For example, Matsura and Nakamura (1981) found that cannibalistic behaviour between same-instar mantids was common when prey availability was low in both high density and low-density experiments. On the other hand, cannibalistic behaviour is still relatively low even at high mantids densities when prey density is low. This data indicates that on average only a few mantids (if any) per ootheca will survive the juvenile stage.

3.3 Mantodea: *Tenodera sinensis*

The Chinese praying mantis is a species native to China and other parts of Asia. It is easily distinguishable from other mantids due to its large size (83-104mm) and colouration of the wings, being brown with a green stripe on the sides. Chinese mantids

have been used for biological control for over 100 years. They were first introduced in North America in 1896 through egg cases and are now commonly found throughout the continental United States and the warmer parts of Canada (Evans, 2007). The Chinese mantis is the subject of the majority of the research conducted on praying mantids due to its abundance and adaptability to the environment. Most of the initial research on mantids was conducted on sexual cannibalism, but now the field has moved more towards an ecological point of view, where mantids are studied for biological control (Hurd, 1999).

4.0 Microsporidia Balbiani, 1882

A great variety of microorganisms can cause infections and diseases in insects. These microorganisms are defined as 'insect pathogens' and can belong to a variety of taxonomical groups, including viruses, bacteria, fungi and protists (Mahr et al., 2008). Insect pathogens can have a negative or positive influence in the environment; some pathogens can affect biological control agents, reducing their efficiency or even killing them, while others may be host specific to a pest and reduce their population. For this reason, there is a growing research on the use of pathogens for microbial control, especially through microbial pesticides (Lacey et al., 2001). Some insect pathogens quickly reduce the population size of the host (like viruses), while others can cause chronic diseases which can affect reproduction, feeding, or development (Tanada &

Kaya, 1993). An example of pathogens causing chronic diseases are microsporidia, which we use in the current study.

Microsporidia are unicellular eukaryotic parasites commonly found in insects, although species have been found in other animal hosts, such as crustaceans, fish and humans (Weber, et al., 1994). Microsporidia have been classified as protozoa, but recent molecular phylogenies arising from the study of the genetic sequence of DNA-directed RNA polymerase II subunit (RPB1) and other proteins indicate that microsporidia did not diverge from early eukaryotes, but instead they share a close relationship to fungi (Hirt, et al., 1999). Even though the taxonomical changes have been accepted, some new literature still ranks microsporidia as protozoa or protists (Becnel & Andreadis, 2014).

Microsporidia are transmitted through spores that are protected by a three-layered cell wall that allows the cell to survive outside a host for several years (Vávra & Larsson, 2014). These pathogens usually enter a host through ingestion, although some species of microsporidia are transmitted from generation to generation through infection of the reproductive organs, as seen in *A. bipunctata* (Steele & Bjørnson, 2014). For microsporidia that are ingested, once the parasite is situated in the gut of an organism, the osmotic pressure inside the spore increases, allowing the internal polar filament to be ejected through the host cell wall into an adjacent host cell. When the polar filament enters the cell of the host's gut epithelium it injects the sporoplasm (infective stage) which eventually produces several nuclei. Each nucleus will then develop into a new spore, which can repeat the cycle and further extend the infection.

Viable spores are transmitted horizontally to autoinfect the same host, or to infect other cells in vulnerable hosts (Louis, et al., 2014). Studies on host specificity of microsporidia have shown these pathogens to be not as host specific as was once thought. Several studies have shown that numerous species of microsporidia are transmissible between different host species, even when these hosts are not closely related (Solter et al.,1997; Saito & Bjørnson, 2008; Solter et al., 2010).

Most microsporidian infections result in long term (chronic) disease which can affect the host in many ways, such as altering mortality, development rate and behavior (Becnel & Andreadis, 2014). For example, the microsporidium *Tubulinosema hippodamiae* is known cause chronic disease in the convergent lady beetle (*Hippodamia convergens* Guérin–Méneville). Infection does not alter the mortality rate, but can significantly slow larval development, as well as hinder female fecundity and longevity (Saito & Bjørnson, 2008).

Although microsporidia are yet to be found in Mantodea, there have been numerous discoveries of these pathogens in insects that are closely related to mantids. Crickets, locusts and grasshoppers are commonly infected with microsporidia that belong to the Genus *Nosema* (Habtewold, et al., 1995). Microsporidia have been widely studied in grasshoppers both in the laboratory and in the field for their potential as microbial biological control agents. For example, infections of microsporidia in the tef grasshopper (*Aiolopus longicornis* Sjöstedt) alter development time, reproductive success, survival and mortality. Some infected individuals display physiological changes,

developing a pink colouration on the abdomen. This change in pattern colouration may be used as a possible indicator of microsporidian infection (Habtewold, et al., 1995). Other studies involving the migratory locust (*Locusta migratoria* L.) indicate that microsporidial infections may result in behavioural changes. In laboratory experiments, high doses of sporal infections cause changes in the morphological transformation of the migratory locust from a solitary phase to a gregarious phase, whereby more individuals remain in the solitary phase of their life cycle. Considering that most of the harm to cultivated crops and other vegetation occurs when the insect reaches its migratory phase, microsporidia infections could be of great advantage to reduce (or even prevent) locust infestations (Fu et al., 2010) .

Nosema adaliae, the pathogen used in this study, is a microsporidian species that was recently discovered to infect *A. bipunctata*. This species has been reported to delay the development of its host larvae by causing chronic disease. *N. adaliae*, like many other microsporidian species, can be transmitted vertically and horizontally, usually through direct consumption (Steele & Bjørnson, 2014). Furthermore, this species has been shown to be able to infect other species within the coleopteran order, such as the seven-spotted lady beetle (*Coccinella septempunctata* L.) (Elkabir, 2016). Considering that *A. bipunctata* is commonly used as biological control agent (Berryman, 1999), it is possible that *N. adaliae* is being introduced accidentally along with its host. Furthermore, some biological control practices involve introducing multiple biological control agents in the same area to further control pest populations. This may allow for

intraguild predation among the introduced organisms, as well as transmission of pathogens, including microsporidia (Symondson et al., 2002).

Study Objectives

The aim of this study is to understand the transmission of the microsporidium *Nosema adaliae* from its natural host, the two-spotted lady beetle (*Adalia bipunctata*) to the Chinese praying mantis (*Tenodera sinensis*). My study will examine the effects of the pathogen on overall development time, intra-molt development, number of failed molts, mortality, and feeding. The objectives of my study are to examine: 1) whether intraguild predation between *A. bipunctata* and *T. sinensis* will allow the transmission of *N. adaliae* from its natural host to *T. sinensis*; 2) if the infection (if present) will cause any effect on the praying mantids; and 3) whether different doses of the pathogen *N. adaliae* will affect the development or mortality of *T. sinensis*. I predict that there will be a successful infection of *T. sinensis* since *N. adaliae* was able to infect other invertebrates beside its natural host (Elkabir, 2016). I also expect an increase in development time and failure of molts, with no change in mortality. This is because similar effects were seen in the natural host of *N. adaliae* (*A. bipunctata*) (Steele & Bjørnson, 2014). Furthermore, these effects were seen in taxonomically closely related host species, such as crickets, grasshoppers, and locusts, which were infected with other species of microsporidia (Fu et al., 2010; Habtewold et al., 1995; Marshall, 2006). Lastly, I expect to observe an

increase in the effects caused by *N. adaliae* when *T. sinensis* consume more infected beetle larvae, as was reported in a previous study on microsporidia (Hembree, 1982).

Material and Methods

Microsporidia-free and microsporidia-infected *A. bipunctata* larvae were reared from laboratory established colonies of adult beetles. Individuals from both uninfected and infected colonies were kept within environmental growth chambers (Sanyo MLR-350H) under controlled conditions (16:8 L:D; 25°C:20°C). Ten mating pairs from both colonies were established and placed in 120 ml clear, polyethylene cups, which were cleaned and bleached prior to use. Uninfected mating pairs were established by using one female and one male *A. bipunctata* from the uninfected stock population, while the microsporidia infected mating pairs were established with one infected female and one uninfected male. Each cup had a 2.2-cm hole in its side that was covered with a fine mesh screen (see Saito & Bjørnson, 2008). Artificial diet (Lacewing and Ladybug Food, Planet Natural, MT) was applied to the side of the cup over part of the mesh. The diet provided nutrients to the beetles and was reapplied when needed. A cotton wick (Crosstex International, NY) was placed inside each cup and moistened daily to allow a water source. Every day, an abundant amount of green peach aphids (*Myzus persicae* Sulzer) was provided. These aphids were reared on nasturtium (*Tropaeolum minus*; Dwarf Jewel Mixed, Stokes Seed Ltd., ON; 16:8 L:D; 25°C:20°C). Two *A. bipunctata* larvae

from each mating pair (uninfected and infected) were randomly examined to confirm the infection status of sibling larvae. Each larva was smeared on a microscope slide and stained with a 5% Giemsa solution (2 h, pH 6.9, Sigma Diagnostics; see Saito & Bjørnson, 2008). The slides were then examined under light microscopy to check the infection status. Infected individuals were identified by the presence of microsporidian spores. Only the living larvae that had a confirmed infection status (through the sibling larvae that were previously smeared) were kept for subsequent use in this study.

Twenty vials (1.25" D × 4" H), each containing 35 microsporidia-free giant flightless fruit flies (*Drosophila hydei* Sturtevant), were obtained from Ward's Science, NY. Flies from each vial were examined for microsporidia by randomly selecting two larvae to be smeared on microscope slides. These were stained with a 5% Giemsa solution. Fruit flies from some shipments were used to establish colonies in the laboratory. Vials of *D. hydei* were stored in an incubator (25°C, dark). Every week, 15 new *Drosophila* culture vials (1.25" D × 4" H; Carolina Biological Supply Company) were prepared for fly rearing. Instant blue medium (Carolina Biological Supply Company) was used as a fly medium and 10 randomly-selected *D. hydei* were placed in each vial. The medium provided moisture and nutrition for both the adults and larvae. The condition of these rearing vials was examined weekly. Vials that did not produce offspring after two weeks, as well as vials that indicated the presence of fungi on the medium, were discarded. Every generation of *D. hydei* that was reared in the lab was examined for microsporidian spores. Three randomly-selected larvae from each vial were smeared on

a microscope slide, stained with 5% Giemsa solution, and examined for microsporidian spores by light microscopy.

Ten microsporidia-free *T. sinensis* oothecae (Natural Insect Control, ON) were placed in 400 ml clear plastic containers (86 mm D x 108.2 mm H; Dollarama) at room temperature. The container lids were equipped with a magnifying lens, which permitted close examination of the oothecae. A cotton wick placed in each container was moistened daily to increase humidity and the oothecae were checked daily for hatch.

Upon hatching, *T. sinensis* nymphs were randomly selected from each egg case. These individuals were assigned to one of three treatments: Control (C), Treatment 1 (T1), and Treatment 2 (T2). The date of hatch was recorded as 'Day 0' and each mantid was coded based on which ootheca it hatched from, the treatment it was assigned to, the mantid number, and hatch date. Newly-hatched mantids were isolated in clear, 120 ml polyethylene cups, similar to the ones made for the *A. bipunctata* mating pairs. All *T. sinensis* nymphs were placed on a tray according to treatment (C, T1, or T2), and all were maintained within an environmental chamber (16:8 L:D; 25°C:20°C). A cotton wick placed in each cup was moistened daily to provide water. Each mantid was fed a specific number of live *D. hydei* daily, accordingly to their instar stage (Table 1). The number of fruit flies consumed was recorded daily and the number of prey available to the mantids was restored daily to the value specified for their specific instar stage.

Table 1. Number of fruit flies (*Drosophila hydei*) fed daily to *Tenodera sinensis* nymphs (according to mantid instar).

Tenodera sinensis instar	Fruit flies fed per day
1st (hatch)	1
2nd	2
3rd	3
4th	4
5th	6
6th	10

Six of ten oothecae hatched (*n* = 24 mantis per treatment; total *n* = 72). For each oothecae, 12 additional mantids were selected at random to be checked for microsporidia infection. Each mantid was smeared on a slide and stained with 5% Giemsa solution. Slides were then checked to determine the infection status.

On Day 15, each mantid in the control and treatment groups was fed one *A. bipunctata* larva, either uninfected (ABU) or infected (ABI), accordingly to the treatment. A second larva was provided on Day 16. Mantids in the control (C) group were provided with two ABU larvae, the ones in Treatment 1 (T1) were given one ABU and one ABI, and the ones in Treatment 2 (T2) were given two ABI. Each mantis was then given 48 hours to consume the larvae. Mantids that did not feed on both *A. bipunctata* larvae, as well as those that died prior to day 15, were removed from the trial and their data was discarded. Mantids that remained in the study were fed daily and observations on health

status, number of flies consumed and molts were reported. Mantids that did not complete their molt were recorded as `failed to molt`.

Mantids that reached the fourth instar were moved to 945 mL, transluecent polyethylene containers (13.5 cm H x 11 cm D, Deli-Pro^{TM}) with lids. Each contained a cotton wick. A 9-cm hole was cut in the lid of each cup, which was covered with a fine mesh screen. The mesh allowed for air circulation and provided a surface for the mantids to hang from when they molted. Mantids were observed until death, at which point the date of death was reported and the individual was tested for microsporidia infection. Deceased mantids were placed on two microscope slides, with the head on one slide and the rest of the body on the other. Slides were then stained with a 5% Giemsa solution and checked for the presence of microsporidian spores by light microscopy.

Results

All *T. sinensis* and *D. hydei* that were examined to confirm infection status prior to the beginning of the study were microsporidia-free. All *A. bipunctata* larvae that were examined to confirm infection status of their sibling (test) larvae confirmed the presence or absence of microsporidia: all ABU larvae were confirmed as microsporidia-free, while all ABI larvae were confirmed as microsporidia-infected. Lastly, all specimens of *T. sinensis* used to check infection status were microsporidia-free. Upon death,

microsporidian spores were not observed in mantids from the three study groups: Control, Treatment 1 and Treatment 2.

Individuals from the control had the lowest mean longevity, 79.45 days. On the other hand, Treatment 1 individuals had a mean longevity of 82.11 days and individuals from treatment 2 had the highest mean longevity of 88.06 days. Although there appears to be a difference between the treatments, these means did not differ significantly. Mantis development data (the number of days each mantis was alive) were tested for normality using the Anderson Darling normality test (RStudio Team 2015) and data were found to not deviate significantly from a normal distribution for all treatments ($p > 0.05$). An ANOVA test (RStudio Team 2015) was used to compare average longevity among treatments. The test generated an F-Value of 0.34 with a corresponding *p*-value of 0.716, indicating the absence of any significant difference in mantids longevity between the treatments (Table 2).

Table 2. ANOVA test results used to compare average longevity among treatments (Control and Treatments 1 and 2).

	N	Mean	St Dev	95% CI	*p*-Value
Control	20	79.45	29.34	(64.72, 94.18)	0.716
T1	19	82.11	34.29	(66.99, 97.22)	
T2	18	88.06	34.99	(72.52, 103.59)	

Mantis feeding data (the number of fruit flies consumed per day) were collected and analyzed starting on Day 17, the day after the mantids were fed *A. bipunctata* larvae. Data were tested for normality using the Anderson Darling normality test (RStudio Team 2015) and data were found to deviate significantly from a normal distribution ($p < 0.05$). Therefore, a Kruskal-Wallis test (RStudio Team 2015) was used to compare the average flies fed per day among treatments. The test generated an H-Value of 21.61 with a corresponding *p*-value <0.0001, indicating a significant difference among at least two of the treatments. Overall, the number of flies eaten differed significantly, with individuals of the control group feeding on significantly fewer prey (3.4 fruit flies per day); than individuals from the two other treatments (4.0 fruit flies per day; Treatments 1 and 2; Table 3). The table indicates a number N, corresponding to the number of days for which the data were collected. The difference in these numbers is due to the different maximum longevity of each treatment group.

Table 3. Kruskal-Wallis test used to compare the average number of fruit flies (*Drosophila hydei*) fed per day among treatments. Reported data collected only after mantids were fed *Adalia bipunctata* larvae on Days 15-16.

	N	Median	Z-Value	H-Value	*p*-Value
Control	122	3.400	-4.62	21.61	0.00002
T1	125	4.000	1.77		
T2	136	4.036	2.77		

A Dunn's test for multiple comparison of independent samples (RStudio Team 2015) was then used to assess the pairwise differences between treatments. A Bonferroni adjustment of the P-Value (RStudio Team 2015) was applied to adjust for the inflation of the Type I error. The Dunn test showed a statistical difference between Control and T1, as well as between Control and T2. Individuals from the control group consumed statistically fewer fruit flies on average compared with Treatment 1 (p = 0.0005) and Treatment 2 (p = 0.00005). Data from Treatments 1 and 2 did not differ significantly (Table 4).

Table 4. Dunn's test for multiple comparison of independent samples with Bonferroni correction used as post hoc test for Kruskal-Wallis test. The test indicated statistical differences between Control and Treatments 1 (T1) and 2 (T2).

	Z-Value	*p*-Value (adjusted)
Control-T1	-3.734	0.0005
Control-T2	-4.300	0.00005
T1-T2	-0.491	1.0

Average flies consumed per day was plotted for all three treatments (Figure 1). The represented data starts on Day 17, the day after the *A. bipunctata* larvae were fed to the mantids (Day 15-16). The three treatment groups follow similar trends at first, but differentiate as time passes. The highest data point (represented by the highest average number of flies consumed in one day) is observed for Treatment 2 (Day 112, average of

9.7 flies consumed). The highest data point for Treatment 1 was on Day 123 (average of 8.0 flies consumed). Lastly, individuals from the Control group consumed the fewest, highest number of flies in one day (Day 120, an average of 7.5 flies). There were several days when individuals of the control group did not consume any flies (Days 127, 130, 134, 135, 136, and 138; the latter was the day the last mantis died). On the other hand, flies were consumed daily by individuals from Treatments 1 and 2. Individuals from these groups did not stop feeding until the day that the last individual from these groups had died, on Days 141 and 152, for Treatments 1 and 2, respectively (Figure 1).

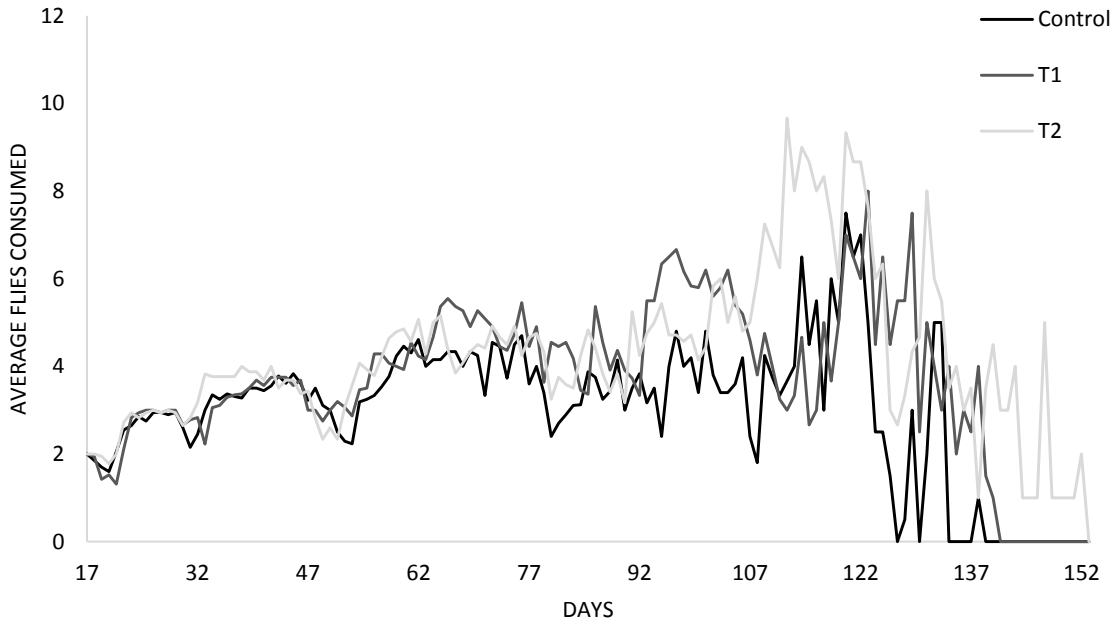

Figure 1. Mean consumption of *Drosophila hydei* consumed per day by *Tenodera sinensis*. Reported data collected only after mantids were fed *A. bipunctata* (on Days 15-16).

Mortality data for each instar were calculated and compared among treatments. Data were converted to percentages and an X^2 test (RStudio Team 2015) was used to test for a statistical difference between mortality rates and treatment. A statistical difference was found between the Control and T1 groups as well as the Control and T2 groups in the third instar. Furthermore, T2 showed statistically fewer deaths than T1 in the fourth instar (Table 5). Overall, mortality did not differ significantly among groups when data were pooled. The control group experienced 70% mortality by the last instar stage, while Treatment 1 and Treatment 2 had 63% and 61% mortality, respectively.

Table 5. Percentage mortality of *Tenodera sinensis* by instar from the three treatment groups (Control and Treatments 1 and 2). First-instar data was excluded *(Adalia bipunctata* larvae were fed to *T. sinensis* nymphs on Days 15 & 16).

Treatment	N	2nd Instar (% deaths)	3rd Instar (% deaths)	4th Instar (% deaths)	5th Instar (% deaths)
Control	20	0	0^{A}	20	70
T1	19	0	5^{B}	26^{A}	63
T2	18	0	6^{B}	11^{B}	61

*Values with different letters indicate the presence of a statistical difference.
*Control treatment showed statistically less deaths than T1 (p=0.024) and T2 (p=0.013) in the 3rd instar.
*T2 showed statistically less deaths than T1 (p=0.006) in the 4th instar.

Percentage of mantids alive versus time (days) for all three treatment groups appears in Figure 2. Each symbol represents the day that each of the mantids died. Treatment 2 has the furthest data point on the X axis (Day 153), indicating the day the

last individual of the treatment died. For the control, the mantid that lived the longest lived until Day 139, represented by the line touching the X axis. The last individual in Treatment 1 died 142 days after the mantids were fed *A. bipunctata* larvae.

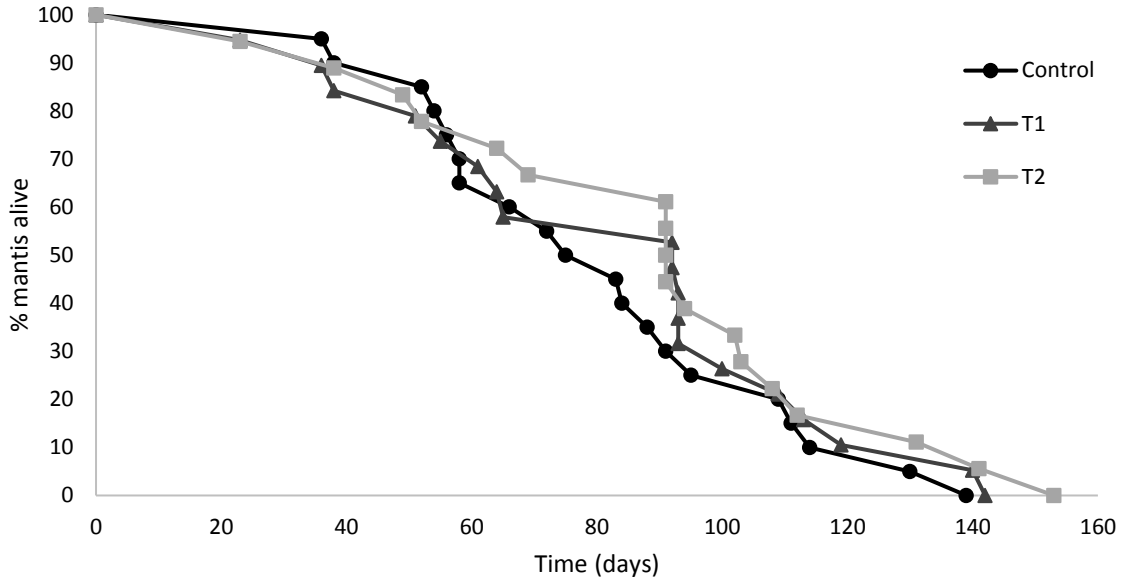

Figure 2. Percentage of *Tenodera sinensis* nymphs alive per day of the trial. Mortality before Day 15 was excluded (*Adalia bipunctata* larvae were fed to *T. sinensis* nymphs on Days 15 & 16).

Data regarding number of failed molts were converted to percentages and compared between treatments. An X^2 test was used to determine statistical difference (RStudio Team 2015). Statistical differences were found between the Control and both Treatments 1 and 2 (*p* values of 0.002 and 0.041, respectively). In the control group, 35%

of the mantids died due to failure in the molting process, whereas 16% and 22% of the mantids failed to molt in Treatments 1 and 2, respectively. (Table 6).

Table 6. Percentage of *Tenodera sinensis* that failed to molt. Data recorded before Day 15 was excluded *(Adalia bipunctata* larvae were fed to *T. sinensis* nymphs on Days 15 & 16).

Treatment	N	Failed Molts (%)
Control	20	35^{A}
T1	19	16^{B}
T2	18	22^{B}

*Values with different letters indicate the presence of a statistical difference.
*Control treatment showed statistically more failed molts than T1 (p=0.002), as well as T2 (P=0.041).

Discussion

The results of this study suggest that the microsporidium *N. adaliae* is unable to infect the Chinese praying mantis. None of the mantids during this study became infected with *N. adaliae*, and there were no statistical differences in mortality, development time, or the mean number of flies eaten among the groups.

1.0 Infection

All the slides prepared to check the infection status of the mantids in my study were observed under light microscopy and all mantid specimens were microsporidia-

free. *N. adaliae* is transmitted horizontally (100% transmission) through consumption of infected host eggs, and this pathogen is able to infect invertebrates other than its natural host (*A. bipunctata*), such as the seven-spotted lady beetle (*Coccinella septempunctata*) (Elkabir, 2016). Therefore, the lack of infection in *T. sinensis* was unexpected. These results may be explained by three different theories: 1) *T. sinensis* presents immunity to microsporidia infections, although there is no evidence to support this assumption; 2) *T. sinensis* has natural immunity to *N. adaliae*, this is likely because the pathogen may be able to infect coccinellid hosts only; and 3) it is possible that *N. adaliae* remained undetected in the samples that were examined.

The possibility that *T. sinensis* is immune to *N. adaliae* is of interest from two points of view. With respect to biological control, the results of my study suggest that the use of *T. sinensis* along with *A. bipunctata* would not result in the transmission of *N. adaliae* from the latter to the former. This would indicate that intraguild predation between the two species would not lead to *T. sinensis* being less effective as a biological control agent due to disease caused by *N. adaliae*. On the other hand, these results suggest that *T. sinensis* may possess a characteristic that makes it immune to *N. adaliae*, and perhaps to microsporidia, although this has not been studied yet. Interestingly, other species that belong to the orthopteroid orders are susceptible to microsporidia. For example, microsporidia within the *Vairimorpha* genus are able to infect the Mormon cricket (*Anabrus simplex* Haldeman) (MacVean & Capinera, 1992), and *N. locustae* is able to parasitize the tef grasshopper (*Aiolopus longicornis*; Habtewold et al., 1995), the

migratory locust, (*L. migratoria*; Fu et al., 2010; Sokolova & Lange, 2002), the South American locust (*Schistocerca cancellata* Stal), the grasshopper *Dichroplus schulzi* Bruner, and many other related species (Sokolova & Lange, 2002). Therefore, understanding what makes this insect immune to *N. adaliae* could provide some insight on how to counteract microsporidia infections in other invertebrates, and perhaps even other organisms belonging to other taxonomic groups.

2.0 Mortality & longevity

Overall, mortality did not differ significantly among the three groups but the control group had the highest percentage of deaths before the last recorded instar, reaching 70%. Treatments 1 and 2 had 63% and 61% mortality rates, respectively (Table 5). Despite the 0% infection rate, none of the mantids reached adulthood and only a small percentage (30% for Control, 37% for T1, and 39% for T2) were able to reach their last larval stage (Table 5). These results cannot be attributed to infection because none of the mantids became infected with *N. adaliae*. One explanation of why so many mantids died during this study can be attributed to their life history. High mortality has been reported in juvenile mantids, whereby only 7% of the juveniles survive per ootheca, even after excluding external factors, such as cannibalism and predation (Hurd & Eisenberg, 1984). Therefore, the high mortality observed during my study may be attributed to mantid biology.

Longevity results were similar to mortality. Individuals in the control group had the lowest mean longevity of 79.45 days, followed by Treatment 1 with 82.11 days, and Treatment 2 with a mean of 88.06 days (Table 2). This could also be explained by the biology of *T. sinensis*. The constant diet of fruit flies that were fed to the mantids in this study could be lacking in adequate nutrition for proper development of a generalist predator like *T. sinensis*, which would normally consume a variety of prey in nature.

3.0 Failed molts & Feeding

Two interesting trends seen in the Control group were the statistically higher number of failed molts (Table 6) and the statistically smaller number of flies consumed (Table 4). Among the control, 35% of the mantids died due to failure in the molting process, whereas only 16 and 22% died during their molts from Treatments 1 and 2, respectively. Furthermore, individuals from the Control group consumed significantly fewer prey (3.4 fruit flies per day); than individuals from the two other treatments (4.0 fruit flies per day; Treatments 1 and 2).

These findings were unexpected since I predicted the opposite trend: an increase in failed molts within the infected treatments (T1 and T2) and greater prey consumption by individuals in the Control group. My predictions were supported by previous studies on species that are susceptible to microsporidia that are also closely related to *T. sinensis*, such as crickets, grasshoppers, and locusts. In these insects, infected individuals

exhibit abnormalities related to the molting process (Fu et al., 2010; Habtewold et al., 1995). For example, microsporidia within the Genus *Vairimorpha* reduce the survival of the Mormon cricket, *A. simplex* by more than 60% during the molting process (MacVean & Capinera, 1992).

4.0 Possible explanations

Since *Nosema adaliae* did not infect any of the mantids in my study, the results cannot be attributed to the effects of microsporidia on the praying mantids. Possible explanations of why statistical differences were observed among the groups include: 1) effects caused by the diet provided to the mantids; 2) *N. adaliae* could have lowered the toxicity of the *A. bipunctata* larvae; and 3) experimental error.

Fruit flies were used as mantid diet because they are common prey for mantids (Elzinga, 1997). Fruit flies are known to host microsporidia (Futerman et al., 2005; Franzen et al., 2005). Futerman et al. (2005) report that microsporidia in *Drosophila* cause pupal mortality in up to 89% of individuals examined. Furthermore, microsporidia infection in fruit flies is associated with a reduction in female fecundity of up to 55%. The same study also determined an infection rate of 100% when transmitted through contaminated food sources. On the other hand, crickets are also known to host microsporidia (Becnel & Andreadis, 2014; Fu et al., 2010; Habtewold et al., 1995). A study on the effects of the microsporidium *Nosema locustae* Canning on the tef

grasshopper (*A. longicornis*) associated infection status with an increase in mortality, with only 19% of the population reaching adulthood.

Giant fruit flies, *D. hydei* were used as food source for the mantids because fruit flies have a quick generation time, which allows a large population of fruit flies to be quickly reared and maintained in the lab. The quick generation time was also a convenient way to test their infection status at each generation, ensuring that the *D. hydei* provided to the mantids were microsporidia-free. On the other hand, fruit flies may have been an insufficient food source for mantid development. The large number of small prey items would require a higher amount of energy for capturing and handling the prey when compared to the access to a single, large one. This could have created a reduction of the benefits versus costs ratio, leading to insufficient nutrition (Griffiths, 1980). This may explain the increase in mortality that was observed in older instars (Table 5). Studies on food limitation in *T. sinensis* have shown a positive relationship between food limitation and increase in mortality, indicating that mortality was statistically higher when a poor diet was provided. Furthermore, the same studies indicate a decrease in growth rate and molting when mantids are reared with reduced food sources (Eisenberg et al., 1981; Moran & Hurd, 1997). Another study has shown a direct relationship between food limitations and increase in percentage of failed molts for *T. sinensis*, which could explain, in part, the results obtained in my study (Eisenberg et al., 1981).

The second explanation of the results obtained in this study may be associated with *A. bipunctata* larvae used as vectors for the transmission of *N. adaliae*. The reason behind the statistically significant differences observed between the control group and the other two treatments (Treatments 1 and 2) may be related to the effects of the microsporidian infection on *A. bipunctata*, more so than the praying mantids. Lady beetles often use alkaloids as chemical defences, which are found to be distasteful or even toxic to other organisms (Daloze et al., 1994). The lady beetle used in this study, *A. bipunctata*, is known to produce large quantities of the alkaloids adaline and adalinine (de Jong, et al., 1991; Daloze et al., 1994). The toxicity of *A. bipunctata* alkaloids could have affected all the individuals in this study, but it is important to keep in mind that the larvae fed to the control group were not infected. We now know that *N. adaliae* causes negative effects on the health status of the host, such as a decrease in development rate, decrease in adult longevity, and reduction in female fecundity (Steele & Bjørnson, 2012). *N. adaliae* reduces the health of infected lady beetles, perhaps hindering their ability to produce alkaloids, thereby reducing their chemical defence. A reduction in adaline and/or adalinine production in microsporidia-infected larvae fed to *T. sinensis* (Treatments 1 and 2) may cause less harm to the mantids feeding on them. If this is true, it could explain the results obtained in this study. Of course, this is sheer speculation.

Lastly, my results are based on a small sample of mantids in three treatments. If the study was to be repeated with a greater number of individuals, different results may be obtained on mortality, longevity, feeding data, and molting success. All we can take

away from this study is that *T. sinensis* was not infected by the microsporidium *N. adaliae*.

Conclusion

Nosema adaliae was unable to infect *Tenodera sinensis* through consumption of lady beetle larvae. Although the pathogen failed to infect the praying mantids used in this study, some statistically significant differences were observed among treatments. Individuals of the Control group were more likely to experience failure of molts and consume fewer prey. Furthermore, individuals from the Control group did not live as individuals from the other two groups, although these observations were not significant. Unfortunately, there are no studies conducted on praying mantids and microsporidia infections. Future studies may provide further insight into my findings.

Although the results of my study did not support my predictions, the results suggested that *A. bipunctata* and *T. sinensis* can be used simultaneously as biological control agents within a localized area without transmitting the microsporidium *N. adaliae*. It is important to keep in mind that intraguild predation may still occur; therefore, using both predators in the same enclosed area may decrease the efficiency of the biological control effort. Future studies that look at prey availability versus intraguild predation will allow us to further understand the benefits versus the costs of this biological control practice.

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