Ultrastructure and tissue pathology of two microsporidia infecting the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae) and the green lacewing, *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae), used for biological control

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A Thesis Submitted to
Saint Mary's University, Halifax, Nova Scotia
in Partial Fulfillment of the Requirements for
the Degree of Masters of Applied Science.

August, 2013, Halifax, Nova Scotia

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Abstract

Two undescribed microsporidia were found in insects that are commercially available for biological control: one from field-collected *Adaliae bipunctata* and another from *Chrysoperla carnea* purchased from a commercial insectary. The objective of this study was to describe both microsporidian pathogens by means of their ultrastructure, tissue pathology and ribosomal RNA gene sequences (molecular characterization). Both microsporidia were closely related to several species within the genus *Nosema* and were given the names *Nosema adaliae* sp. nov. and *Nosema chrysoperlae* sp. nov., respectively. Spores of *N. adaliae* measured 4.25 ± 0.09 x 1.82 ± 0.03 μm and had an isofilar polar filament with 10-18 coils. Various host tissues were infected, including the fat body, muscles, ovaries, testes, midgut, hindgut and ventral nerve cord. Spores of *N. chrysoperlae* measured 3.49 ± 0.10 x 1.52 ± 0.05 μm and had an isofilar polar filament with 8-10 coils. The majority of host tissues were infected except for the ovaries.

August 2nd, 2013

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Acknowledgements

I would like to thank my supervisor, Dr. Susan Bjørnson, and my thesis committee members for their guidance. I also thank Qianni Hu, Bryan Ellis and Cynthia Leggiadro for their technical support. I am grateful for the sacrifices and support my family and friends have provided over the last two years. I wouldn't have been able to complete this project without the encouragement of my supportive parents, Coleen and Bill Steele. Funding was provided by NSERC (Discovery Grant, SB), the Canadian Foundation for Innovation (CFI New Opportunities Fund) and Saint Mary's University.

INTRODUCTION

The benefits of using insects for the biological control of pests has been known for centuries with many species now commercially available for use in biological control programs. However, an emerging concern with regards to the overall success of biological control is the quality and condition of these natural enemies (van Lenteren, 2003). It is well known that numerous beneficial arthropods host a wide variety of symbionts such as parasites, viruses, eugregarines, fungi, bacteria, nematodes and microsporidia which may be inadvertently imported and released when beetles are used in biological control programs.

Several microsporidian pathogens have been described from beneficial insects used in biological control programs. Microsporidia were once though to be host specific and species were identified on the basis of their host. However, they are now known to infect more than one host, which has lead to taxonomic confusion (Tanada & Kaya, 1993). With this knowledge, it is crucial to examine other characteristics, such as pathogen ultrastructure, tissue pathology and ribosomal RNA gene sequences when describing a new species.

Introduction to biological control

Historically, there have been many different approaches to alleviate insect pests, including the application of chemical pesticides. Chemical controls became commonplace following WWI when various organochlorine, organophosphate and carbamate pesticides

were used in an attempt to eradicate pest populations from a variety of crops (Pedigo & Rice, 2006). However, the overuse of chemical pesticides has resulted in an "evolutionary arms race"- an evolutionary struggle whereby pests develop adaptations as a means to overcome chemical pest control measures. Pesticide-tolerant individuals survive the application of the pesticide and reproduce, thereby increasing the number of tolerant individuals within the pest population (Georghiou, 1972; Liptak, 1995).

In recent years, integrated pest management (IPM) has become the most popular and successful approach for pest control in commercial cropping systems (Pedigo & Rice, 2006). The object of IPM is to reduce economic losses caused by pests in ways that are effective, economically sound and ecologically sustainable. IPM is a control method that incorporates several techniques, including mechanical controls, biological controls and the application of chemical pesticides (Pedigo & Rice, 2006).

Many pests are maintained at low population levels in nature (natural pest control) and as a result, they cause insignificant economic damage to crops or livestock (DeBach & Rosen, 1991). Natural pest control is achieved by the interaction of many abiotic and biotic factors, including the action of natural enemies (parasites, predators, or pathogens) that either kill or decrease the reproductive potential of pest species. Natural enemies are of great biological importance in pest management because they are often host specific and very effective (Flint *et al*., 1998).

Biological control is a type of natural control that involves the use of mass-produced or field-collected natural enemies for pest control in agroecosystems (Perdikis *et al*., 2008). There are three main types of biological control: augmentation, conservation

and classical. The objective of augmentative biological control is to increase the number of natural enemies that already exist in a particular area by either releasing large quantities of the same natural enemies or by modifying the local environment to naturally increase the number of natural enemies present and increase their effectiveness (DeBach, 1964; Pedigo & Rice, 2006). Maintaining the rearing facilities required for the production of large numbers of natural enemies is often expensive; however the inclusion of conservational techniques can reduce the cost (Dixon, 2000).

Conservation biological control focuses on the protection of existing populations of natural enemies by protecting the local environment within an ecosystem where natural enemies reside (Pedigo & Rice, 2006). Some conservational practices include the modification of traditional agricultural practices to protect local populations of natural enemies (Pedigo & Rice, 2006).

Classical biological control involves foreign exploration and introduction of natural enemies for control of local pest populations (Flint *et al*., 1998). Classical biological control is effective when the pest species has been introduced, inadvertently or otherwise, into a new ecosystem where their indigenous natural enemies do not exist (Flint *et al*., 1998; Pedigo & Rice, 2006). Several steps are required to implement successful importation: (1) identification of the natural pest habitat; (2) search of the native pest habitat for natural enemies; (3) identification of natural enemies and importation for testing; (4) quarantine of natural enemies before release; and (5) mass production of natural enemies and their release into affected area (Flint *et al.*, 1998). Initiating a classical biological control program may be time-consuming and expensive

and success is dependent on obtaining effective natural enemies from their areas of origin (Dixon, 2000). Once established, classical biological control may be cost-effective and self-sustaining (Dixon, 2000).

History of biological control

Over the last hundred years, biological control has rapidly evolved and increased in popularity due to advances in modern agriculture (DeBach, 1964). The earliest known record of biological control dates back to fourth-century China where ants were used to control citrus pests (Huang & Pei, 1987; McCook, 1882). Farmers placed nests of Asian weaver ants, *Oecophylla smaragdina* F., in mandarin orange trees to reduce the numbers of the foliage-feeding insect, *Tessaratoma papillosa* (Drury) (McCook, 1882). Interconnecting bamboo rods were installed as bridges to assist the ants in traveling from tree to tree (Huand & Pei, 1987). This represents the first known case of conservational biological control (Ehler, 1998).

The use of ants in fourth-century China was first documented by Rev. Dr. Henry McCook, an American Presbyterian clergyman and naturalist, in 1882. McCook realized the importance of beneficial insects for pest control and was inspired by his knowledge of Asian weaver ants to question if other insects (including North American ants) possessed similar beneficial qualities. He concluded: “whatever benefits the ant may be led by domestication to confer upon man, she already is entitled to consideration as a valuable, if not valued, friend of the race” (McCook, 1882). McCook recommended that the government investigate the potential of beneficial insects for use in biological pest

control. Although McCook documents the first use of biological control in fourth-century-China, he was not the first person to suggest the use of living insects as pest control agents.

The first statement of rationale behind biological control was made in 1800 by Erasmus Darwin in his publication entitled *Phytologia; Or the Philosophy of Agriculture and Gardening*. In a chapter entitled *Plant Diseases* Darwin noted: "The history of the [aphid] is so curious, the destruction it commits on the foliage of the peach tree and nectarine is in dry summers so irresistible, and its existence on other trees so extensive, that it demands our particular attention" (Darwin, 1800). Based on his own observations, Darwin suggested that: "it is plausible, that this plague of the aphid might be counteracted by the natural means of devouring one insect by another". He documented that when hoverflies deposit their eggs where aphids are abundant and as soon as the larvae emerge, they devour hundreds of aphids around them. Based on this discovery, he concluded that hoverfly eggs could be collected and kept overwinter to use in the following spring to eliminate aphid problems in greenhouses. Darwin did a considerable amount of research on plants and their diseases and while studying plant pests, he was the first to observe lady beetles feeding on aphids: "another enemy to the [aphid] is said to be a beautiful small spotted beetle, called a lady-bird by the people". He made note that lady beetles appear in their larval state to feed on aphids, then change to a chrysalis and lastly to a small wing-sheathed beetle (the sheathed wings he believed were a protective covering for burrowing into the ground).

In 1818, Kirby and Spence discussed the desirability of increasing the number of lady beetles for effective aphid control. They noted that in many countries where lady beetles are in large enough numbers to be considered an annoyance, they are protected because of their ability to save crops. Hops growers often employed children to guard lady beetles in their fields from birds. In France, lady beetles were so praised for their pest controlling abilities that they were regarded as sacred to the Virgin Mary and called Vache à Dieu (God's Cows). Kirby and Spence claimed that "if we could but discover a mode of increasing these insects at will, we might not only, as Dr. Darwin has suggested, clear our hot-houses of aphids by their means, but render our crops of hops much more certain than they now are… [but] even without this knowledge, nothing is more easy, as I have experienced, then to clear a plant or small tree by placing upon it several larvae of Coccinellae or aphidivorous flies collected from less valuable vegetables" (Kirby & Spence, 1818).

Although the effectiveness of lady beetles and other natural enemies was known, biological control did not become an established practice in North America until the late 1800s (Pedigo & Rice, 2006). In 1878, the California citrus industry was still in an early stage of development and young trees were threatened by a massive infestation of cottony-cushion scale, *Icerya purchasi* Maskell. These pests had been introduced to California from Australia in 1868 when infested acacia trees imported by a sugar refiner were planted in Menlo Park, in Palo Alto (Royal Gardens, 1889; Riley, 1893). *I. purchasi* has a high reproductive capacity, is capable of self-fertilization, and has three generations per year under typical California conditions, enabling this pest to establish quickly from

very few individuals (Caltagirone & Doutt, 1989). Cottony cushion scale spread rapidly across the state and with no successful means of control, it was recommended that any infected trees should be immediately cut down and burned (Royal Gardens, 1889). Damage was so extensive that farmers were forced to abandon their commercial citrus groves. Cottony-cushion scale was rated one of the most destructive pests (Riley, 1893) and it was believed that no human endeavor could exterminate it (Royal Gardens, 1889).

California State entomologist Charles Valentine Riley determined the countries of origin of the cottony-cushion scale and teamed up with fellow entomologist Arthur Koebele to search for natural enemies in Australia and New Zealand. Koebele, after arriving in Australia in September of 1888, quickly started looking for evidence of scale on trees and talked to local officials to gather information about cottony-cushion scale. He soon discovered that cottony cushion scale was not extensively injurious in many parts of Australia, taking note that "occasionally and in certain spots they became numerous, but always disappeared again" (Koebele, 1890). While staying in Adelaide, Koebele observed numerous aphid and scale predators including a parasitic fly and a lady beetle feeding on the cottony-cushion scale (Koebele, 1890).

The vedalia lady beetle, *Rodolia cardinalis* (Mulsant) and a parasitic fly, *Cryptochaetum iceryae* (Williston) were imported by steam ship from New Zealand and Australia to California in cooled, wooden boxes with scale-infected branches (Koebele, 1890; Riley, 1893). Once received, the beetles were placed under a tent on an *I. purchasi*-infested orange tree and allowed to breed undisturbed (Caltagirone & Doutt, 1989). One side of the tent was removed to allow the beetles to spread to adjoining trees. This served

as the first insectary in California (Caltagirone & Doutt, 1989). Koebele recognized that the vedalia beetle showed great potential for scale control. He wrote: “I shall be greatly mistaken if this one insect alone is not master of the situation within two years’ time”. The vedalia beetle proved to be very successful “as to throw the others entirely into the shade and render their services really unnecessary”. The cottony-cushion scale was completely controlled by the vedalia beetle within one and a half years following its introduction (Koebele, 1890; Riley, 1893). More than 100 years after its introduction to California, the vedalia beetle continues to regulate cottony-cushion scale at levels that are extremely low (Caltagirone & Doutt, 1989). Riley concluded the experiment was so striking and important to the California citrus industry that it helped change the mindset regarding pest control and “that our best hope for [pest] destruction lies with the parasitic and predaceous species, not to mention fungus and bacterial diseases” (Riley, 1893). Due to the overwhelming success of Riley and Koebele, the use of lady beetles and other beneficial arthropods for the control of introduced pest species sparked widespread interest and consequently the large-scale commercialization of insect natural enemies increased in Canada, the United States and Europe (van Lenteren, 2003). It is often stated that lady beetles are responsible for the development of modern biological control programs (DeBach, 1964) and Charles Valentine Riley is often recognized as the “Father of Biological Control” (Dixon, 2000).

Farmers and agricultural officials had become such enthusiastic supporters of biological control that a worldwide infatuation with lady beetles, referred to as the “ladybird fantasy”, quickly took over the pest management industry (Caltagirone &

Doutt, 1989). By the 20th century, protocols had become established for the collection and distribution of lady beetles for biological control. In the early 1900s, convergent lady beetles (*Hippodamia convergens* Guérin-Méneville) became instrumental for the control of melon aphid, a pest that had devastated field crops in California. In his article entitled *Collecting Lady Beetles by the Ton*, Carnes describes the process of climbing the Sierra Mountains in mid-January to collect hibernating convergent lady beetles beneath the snow (Carnes, 1912). Adults were dug up by hand, placed in sacks and transported down the mountain by mule. Beetles were later sorted and packed in colonies of roughly 33,000 individuals for redistribution to farmers. This was the earliest recorded example of augmentative biological control in North America. Today, convergent lady beetles are still collected and redistributed throughout North America for aphid control on agricultural crops and in home gardens (Obrycki & Kring, 1998).

Augmentative biological control increased in popularity when the endoparasitoid, *Encarsia partenopea* proved effective for greenhouse whitefly (*Trialeurodes vaporariorum*) control in the 1920s (Hussey & Scopes, 1985). Entomologist G. Fox-Wilson at the Royal Horticultural Society Gardens in England realized that mass releases of *E. partenopea* could be used to control whitefly outbreaks in greenhouses (Gurr *et al*., 2000). Within a decade, commercial production of *Encarsia* began in Britain (Hussey & Scopes, 1985; Gurr *et al*., 2000). Today, several species of natural enemies, including lady beetles, are commercially available for augmentative biological control.

Natural enemies: Adalia bipunctata & Chrysoperla carnea

As of 1990, 63 predatory insect and mite species had been released to control 722 species of introduced plant pests in the United States (Pedigo & Rice, 2006). The two-spotted lady beetle, *Adalia bipunctata* L. and the green lacewing *Chrysoperla carnea* Stephens are two natural enemies that are commercially available for pest control in biological control programs.

The two-spotted lady beetle, *A. bipunctata*, is a tree-dwelling aphidophagous ladybeetle endemic to Europe, Central Asia and North America (Majerus, 1994). Adults exhibit polymorphic colorization that occurs in various forms and is controlled by multiple allelic series (Hodek & Honěk, 1996). There are two main forms of colorization: melanic and non-melanic. The most common melanic form is black with four to six red spots and the most common non-melanic form is red with two black spots (Hodek & Honěk, 1996; Pervez & Pervez, 2005). The green lacewing, *C. carnea* is a generalist predator with a wide geographic distribution including parts of North America, Europe and Asia (Pedigo & Rice, 2006). Adults have a green cylindrical body with light green veins on sizeable, transparent wings and golden colored eyes (Pedigo & Rice, 2006).

The life cycle of *A. bipunctata* is similar to that of other lady beetles. The life cycle begins when eggs (usually laid as part of a cluster) hatch to give rise to larvae that develop through four instars (Dixon, 2000). The life cycle of *C. carnea* is similar to that of *A. bipunctata*: eggs hatch into larvae, which undergo four instars before pupating to eclose as adults (Pedigo & Rice, 2006). However, green lacewings eggs, unlike those of other insects, are each laid atop a stalk that elevates it above the leaf surface to provide

protection from cannibalistic sibling larvae. Fourth instar-larvae spin a protective cocoon before pupation (Pedigo & Rice, 2006).

Two-spotted lady beetles consume a wide range of aphid prey along with coccids and diaspids (scale insects) as alternative prey and feed on pollen when aphids are absent or scarce (Hemptinne & Desprets, 1986). Green lacewing larvae consume a wide variety of soft insects and eggs but they also feed on any prey that are similar in size or smaller (Pedigo & Rice, 2006). Adult lacewings feed primarily on honeydew (Pedigo & Rice, 2006). Cannibalism is fairly common in the insect world (Van Driesche *et al.,* 2008), especially among lady beetles (Pervez & Pervez*,* 2005). Studies have shown that *A. bipunctata* have high rates of inter- and intra-guild egg cannibalism that is thought to be advantageous for larval development and can increase survival of sibling larvae when food is scarce (see Burgio *et al.,* 2002; Roy *et al.,* 2007). Cannibalism is also well documented for *C. carnea* but is considered a natural tendency of this generalist predator (Duelli, 1981; Canard & Duelli, 1984).

Both *A. bipunctata* and *C. carnea* can grow and develop on suitable prey under controlled conditions and both consume a large amount of prey, making them perfect candidates for mass-production and biological control. The two-spotted lady beetle has been commercialized for aphid control in Europe since 1999 and was used in biological control programs in North America shortly thereafter (De Clercq *et al*., 2005). Past attempts at establishing the two-spotted lady beetle was unsuccessful until *A. bipunctata* eggs and larvae were used to control the rosy apple aphid, *Dysaphis plantaginea* (Passerini) in Switzerland (Pervez & Pervez*,* 2005; Wyss *et al*., 1999). Green lacewings

were first noted as a potential biological control agent by Albery Koebele in 1888 (Koebele, 1890). Koebele, while searching for a natural enemy to control cottony cushion scale, observed green lacewings feeding on scale and shipped specimens back to California for examination (Koebele, 1890). Since then, mass-culturing techniques have been developed and *C. carnea* eggs and first instar larvae are sold for greenhouse and field applications (Finney, 1950; McEwen *et al*., 2001; Ridgway & Murphy, 1984)

Lady beetles have very few natural predators but are known to be hosts to numerous symbionts and most of these are known to infect *A. bipunctata*. The list of parasitoids and pathogens that affect lady beetles includes flies (*Phalocrotophora* spp., *Medina* spp.), wasps (*Dinocampus coccinellae*, *Homalotylus* spp., *Tetrastichinae* spp., *Pediobius foveolatus*), mites and ticks (*Coccipolipus* spp.), nematodes (*Parasitilenchus coccinellinae*, *Howardula* spp., *Mermitidae* spp.), protozoans (gregarines), fungi (*Hyphomycetes,* microsporidia), male-killing bacteria (*Wolbachia, Rickettsia* and *Spiroplasma*) and several viruses (see Richerson, 1970; Hodek & Honěk, 1996; Riddick *et al*., 2009). Lacewings are well known for their symbiotic relationships with beneficial microbes (particularly yeasts, bacteria and fungi) as opposed to their relationships with parasites and pathogens; however, the role of these symbiotic microbes is not fully understood (New, 1975).

Microsporidia

Microsporidia are small, obligate intracellular eukaryotic pathogens that produce unicellular spores that are involved in transmission (Bulla & Cheng, 1976; Tanada &

Kaya, 1993). Microsporidia are highly specialized to depend on their hosts and are characterized by a dramatic reduction of cellular components (Keeling & Fast, 2002; Texier *et al*., 2010). The evolved dependence between microsporidia and their hosts has resulted in a reduction of pathogen morphology, organelles, biochemistry, metabolism and genomes (Keeling & Fast, 2002).

The simplistic cellular structure of microsporidia has made it difficult to determine the evolutionary relationship of this pathogen to other eukaryotes, since they lack many characteristics historically used to make comparison (Corradi & Keeling, 2009). Microsporidia were originally described as a yeast-like fungus; however, the unique mode of infection led researchers to separate them from fungi (Keeling & Fast, 2002). As knowledge increased on the diversity of microbial eukaryotes, microsporidia were grouped with other spore-forming parasites called Sporozoa (Keeling & Fast, 2002). The taxonomic classification of microsporidia was changed numerous times, but developments in molecular technology resulted in microsporidia being classified as a fungus once again (Corradi & Keeling, 2009). Genomic work suggests a relationship to fungi, however, the exact relationship has yet to be determined (Keeling & Fast, 2002).

Microsporidia infect a large range of hosts, including all five classes of vertebrates, most invertebrate phyla and some protists (Bulla & Cheng, 1976). Over 1300 species of microsporidia have been found within 160 genera (Lacey & Kaya, 2007); however, recent work suggests the number of genera has increased to 198 (J. J. Becnel, personal communication) . The most common hosts are arthropods. Microsporidia are important pathogens of insects and can cause economically serious diseases

(microsporidiosis) in both beneficial and pest insect species (Tanada & Kaya, 1993). Microsporidia were once thought to be host specific and were identified on the basis of their host. This has caused some taxonomic confusion because some microsporidia are now known to infect more than one host (Tanada & Kaya, 1993).

Microsporidia infect a new host with penetration structure, unique to this phylum, called a polar filament (Tanada & Kaya, 1993). The mode of polar filament extrusion and what triggers this reaction is still not fully understood (Tanada & Kaya, 1993). After appropriate environmental stimuli, such as a change in pH or osmotic pressure, the posterior vacuole swells to increase internal pressure inside the microsporidian spore, which causes the polar filament to rapidly discharge. During this process, the polar filament everts to form a hollow tube that works like a needle to inject the infective material (sporoplasm) into the new host cell (Keeling & Fast, 2002; Tanada & Kaya, 1993; Xu & Weiss, 2005). The interaction of a microsporidium within a host cell depends greatly on the host organism, but within insect cells, the microsporidium typically develops under favorable conditions and spreads without limitation throughout the entire cytoplasm, eventually destroying the cell (Tanada & Kaya, 1993).

The microsporidian lifecycle has two distinct phases: (1) merogony, the vegetative stage that involves the production and multiplication of meronts and (2) sporogony, the production of transmissible spores (Tanada & Kaya, 1993). Once the infective material, or sporoplasm, is injected into a new host cell it grows and develops cytoplasmic organelles and becomes a meront (Keeling & Fast, 2002; Tanada & Kaya, 1993). Meronts are non motile cells that are typically round with one or two compact round

nuclei (Solter & Becnel, 2007). Meronts divide rapidly to start a new merogonial cycle, either by binary fission or by nuclear division that results in a plasmodium (Tanada & Kaya, 1993). At a certain stage in the cycle, the meront divides into a sporont (Keeling & Fast, 2002; Tanada & Kaya, 1993). These resemble meronts but can be easily distinguished by the structure of their plasma membrane, which start to thicken at this stage, the development of endoplasmic reticulum around the nucleus and an increase in the number of ribosomes (Keeling & Fast, 2002; Tanada & Kaya, 1993). Sporonts may divide once, to form a sporoblast, or several times to form more sporonts which then divide into sporoblasts (Tanada & Kaya, 1993). The sporoblast is a clearly defined stage that precedes the spore. At this stage, the polar filament, polaroplast, and posterior vacuole begin to form (Keeling & Fast, 2002; Tanada & Kaya, 1993). Once the sporoblast reaches maturity, the cell decreases in size and the endospore layer develops revealing a mature spore (Keeling & Fast, 2002).

Microsporidian life cycles vary greatly in their complexity and transmission routes. The life cycle of some species are direct with a single spore type whereas others are indirect and may have up to four different spore types, numerous divisions throughout the developmental stages or require an intermediate host (Solter & Becnel, 2007; Tanada & Kaya, 1993). The means by which microsporidia spread throughout an infected individual has not been fully established. There are two possible theories explaining the spread of vegetative stages: (1) meronts are transported by phagocytic cells that incorporate them into host tissues; and (2) degenerated cells release vegetative forms enclosed in vesicles (Tanada & Kaya, 1993). The most well established mechanism for

the spread of microsporidia within an individual is the production of early spores, whose primary role is to infect secondary cells within the same host (Agnew *et al.*, 2003). This process is referred to as autoinfection. Early spores are more economical to manufacture in terms of time and material due to their shorter polar filaments and thinner spore walls (Agnew *et al.*, 2003).

Microsporidia are transmitted through three natural portals of entry: oral, cuticular, and ovarial (Kramer, 1976). Transmission by the oral and cuticular pathways results in horizontal transmission (from one individual to another within a population) whereas vertical transmission (from parent to progeny) is achieved through the ovarial portal (Tanada & Kaya, 1993). Insects infected with microsporidia may be altered in colour, size, form and activity, depending on the organs or tissues infected; however, infected insects may not be observably altered (Tanada & Kaya, 1993). Infected larvae often exhibit delayed growth, reduced size prior to death, and reduced activity levels (Tanada & Kaya, 1993). Other symptoms include abnormal feeding, irregular growth, incomplete metamorphosis, deformed pupae and adults, and lowered fecundity (Kluge & Cladwell, 1992).

Several microsporidia have been described from natural enemies available for biological pest control, including the predatory mites *Phytoseiulus persimilis* Athias-Henriot (Bjørnson et al. 1996; Bjørnson, and Keddie, 2000), *Neoseiulus cucumeris* Oudemans and *N. barkeri* Hughes (Beerling and van der Geest, 1991), *Metaseiulus occidentalis* (Nesbitt) (Becnel et al. 2002) as well as the pteromalid endoparasitoid *Muscidifurax raptor* Girault and Saunders (Geden et al. 1992) and the convergent lady

beetles, *Hippodamia convergens* Guérin-Méneville (Bjørnson et al. 2011). Two unidentified microsporidia were recently found infecting field-collected *A. bipunctata* (from local populations in Halifax, NS) and *C. carnea* purchased from a commercial insectary in Europe. The objective of this study is to provide a formal description of these two pathogens by means of their ultrastructure, tissue pathology and ribosomal RNA gene sequences (molecular characterization).

Chapter One

Ultrastructure and molecular characterization of the microsporidium, *Nosema adaliae* sp. nov., from the two-spotted lady beetle, *Adalia bipunctata* L. (Coleoptera: Coccinellidae)

1.1. Introduction

The two-spotted lady beetle, *Adalia bipunctata* L., is a tree-dwelling aphidophagous lady beetle endemic to Europe, Central Asia and North America (Majerus, 1994). *A. bipunctata* consume a wide range of aphids along with coccids and diaspids (scale insects) as alternative prey but they also feed on pollen when aphids are absent or scarce (Hemptinne and Desprets, 1986). The two-spotted lady beetle has been commercialized for aphid control in Europe since 1999 and was made available for biological control in North America shortly thereafter (De Clercq et al., 2005). Early attempts at establishing two-spotted lady beetles for biological pest control were unsuccessful until *A. bipunctata* were used to control the rosy apple aphid, *Dysaphis plantaginea* (Passerini) in Switzerland (Wyss et al., 1999).

An emerging concern with regards to the overall success of biological control is the quality and condition of the biological control agents that are used (van Lenteren, 2003). Lady beetles are known to host a wide variety of symbionts including parasitoids, viruses, eugregarines, fungi, bacteria, nematodes and microsporidia (see Richerson, 1970; Hodek and Honěk, 1996; Riddick et al., 2009). The most studied symbionts in *A. bipunctata* are maternally inherited, male-killing bacteria that are known to reduce egg hatch and result in female-biased sex ratios (Hurst et al., 1996; 1999).

Microsporidia are common pathogens of lady beetles. Four species of microsporidia have been described from field-collected coccinellids, including *Nosema hippodamiae* from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville (see Lipa and Steinhaus, 1959); *N. tracheophila* from the seven-spotted lady beetle, *Coccinella septempunctata* L. (Cali and Briggs, 1967); *N. coccinellae* from several lady beetle species, including *C. septempunctata* (see Lipa 1968; Lipa et al., 1975); and *Tubulinosema hippodamiae* from *H. convergens* (Bjørnson et al., 2011). Two of the four species of microsporidia also infect *A. bipunctata*: *N. coccinellae* infects field-collected beetles in Poland (Lipa, 1968; Lipa et al., 1975) whereas *T. hippodamiae* has been transmitted from *H. convergens* to *A. bipunctata* under laboratory conditions (Saito and Bjørnson, 2008).

Recently, an undescribed microsporidium was isolated from local populations of *A. bipunctata* that were collected in Nova Scotia, Canada. The pathogen delays larval development but has no observable effect on adult fecundity or longevity (Steele & and Bjørnson, 2012). The objective of this study was to formally describe this microsporidium by means of its ultrastructure, tissue pathology and molecular characterization.

1.2. Materials and Methods

Microsporidia-infected *A. bipunctata* adults used in this study were isolated from laboratory-reared colonies established from field-collected individuals. Uninfected *A. bipunctata* were isolated from a single shipment of 100 larvae that were obtained from a commercial insectary.

Adult beetles were kept in 120 ml clear, polyethylene cups (Canemco-Marivac Inc., QC) under controlled conditions (16:8 L:D; 25°C:20°C) within environmental growth chambers (Sanyo MLR-350H). Each cup had a 2.2-cm hole cut in the side, which was covered with a fine mesh screen (80 microns) to allow air circulation. Beetles were maintained on green peach aphids (*Myzus persicae* Sulzer) and artificial diet (Lacewing and ladybug Food, Planet Natural, MT). Distilled water was provided on a moistened cotton wick (Crosstex International, NY). Green peach aphids were reared on nasturtium (*Tropaeolum minus*) (Dwarf Jewel Mixed, Stokes Seed Ltd., ON) in separate environmental chambers under controlled conditions (16:8 L:D; 25°C:20°C).

To confirm the infection status of the beetles used, sibling eggs from each female parent were randomly selected for microscopic examination. Eggs were smeared on microscope slides, stained with a 5% Giemsa solution (2 h, pH 6.9, Sigma Diagnostics), and examined for presence or absence of microsporidian spores by light microscopy.

1.2.1 Pathogen Ultrastructure

Microsporidia-infected *A. bipunctata* adults (n = 48) were randomly chosen to be processed for transmission electron microscopy (TEM). Each beetle was submerged in fixative (2.5% glutaraldehyde) and its head, thorax, elytra, wings and legs were removed. Additional incisions were made in the abdominal sternites to maximize penetration of the fixative. Samples were kept in fixative at 4°C until they were processed (48 h). Sixteen specimens were processed simultaneously in three batches on separate days.

Tissues were embedded in Jembed 812/Spurr resin (Canemco-Marivac, CA) according to the procedure by Becnel (1997, see Appendix A) with the following modification: tissues were placed under vacuum (15 PSI) overnight once they were in pure resin. Tissue blocks (n = 6) were chosen at random for examination by TEM. A Leica UCT ultramicrotome was used to cut ultra-thin sections (~70 nm thick), which were subsequently stained with uranyl acetate and led citrate prior to viewing.

Micrographs were digitally generated with a GATAN ES500W Erlangshen CCD camera side mounted to a Hitachi H7500 transmission electron microscope at 80 kV. Imaging software (ImageJ, 1.45s) was used for determining spore measurements.

1.2.2 Tissue Pathology

Sixteen uninfected and microsporidia-infected beetles were embedded for histological examination. A total of 12 individuals were examined (4 uninfected and 8 microsporidia-infected). Beetles were submerged in Carnoy's fixative (60 ml ethanol, 30 ml chloroform, 10 ml glacial acetic acid) and the head, thorax, elytra, wings and legs of each specimen were removed. Incisions were made in the abdominal sternites to enhance penetration of the fixative. Samples were kept in fixative at 4°C until they were processed (48 to 72 h).

Tissues were embedded in Paraplast® Plus (Sigma-Aldrich Inc., melting point 56°C) according to the procedure by Becnel (1997, see Appendix B) with the following modifications: (1) tissues remained submerged in 1:1 ethanol:butanol overnight in a >25.5°C oven (instead of 2h at room temp); (2) tissues were submerged in 3:1

butanol:paraffin for 20 min in a 60°C oven (this step was inserted between 100% butanol and 1:1 butanol:paraffin); and (3) tissues remained in 1:1 butanol paraffin overnight (instead of 2 h).

Specimens were sectioned longitudinally (5 µm thickness) onto glass slides using a rotary microtome. Slides were coated with a protein solution (1 g gelatin, 2 g solid phenol, 15 ml glycerin, 100 ml distilled water) to help tissues adhere to the slides. Once dry, the paraffin was removed in xylene and sections were rehydrated in solutions made of decreasing ethanol concentrations, ending in distilled water. Sections were stained with Harris hematoxylin and Alcoholic Eosin Y (Fisher Scientific, see Appendix C) and examined by light microscopy. Images of spores and infected tissues were obtained using Zeiss Axiovision imaging software.

1.2.3 Molecular Characterization

Microsporidia genomic DNA was extracted from randomly selected *A. bipunctata* eggs (24 to 48 h old) according to the methods described by Bjørnson et al. (2011). DNA was isolated and purified with a DNeasy® Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. Microsporidian primers described by Vossbrinck et al. (2004) were used to design primers for this study (18f (CACCAGGTT GATTCT GCC)/1492 (GGTTACCTTGTTACGACTT); Eurofins Laboratories). DNA was amplified by Polymerase Chain Reaction (PCR), cloned using pGEM®-T and pGEM®-T Easy Vector Systems (Promega Corp.), extracted with PureYield™ Plasmid Miniprep System (Promega Corp.), and sent to Macrogen (Korea) for sequencing.

The top 10 most closely related species were selected for molecular comparison according to their maximum identity score (Table 1). Sequences of *Nosema bombi, N. thomsoni, N. vespula, N. oulemae, Oligosporidium occidentalis, N. portugal, Vairimorpha lymantriae, N. carpocapsae, V. necatrix* and *N. necatrix* were obtained from NCBI GenBank. *N. apis* was used for comparison as an out group (in an unrooted tree, *N. apis* was distant from other sequences). Automated sequence outputs were visually inspected with chromatographs. Multiple sequence alignments were made using ClustalX with default parameters and additional manual edits to minimize gaps (Thompson et al., 1997). Phylogenetic analysis using the maximum-parsimony (MP) method was performed with the computer program PAUP* ver. 4 beta 10 (Swofford, 2003). All characters were specified as unweighted and unordered, and gap-only columns were excluded in the analyses. The most parsimonious trees were constructed by performing a heuristic search using the Tree Bisection-Reconnection (TBR) with the following parameters: MulTrees on and ten replications of random addition sequences with the stepwise addition option. Multiple parsimonious trees were combined to form a strict consensus tree. Overall character congruence was estimated by the consistency (CI), and the retention index (RI). To infer the robustness of clades, bootstrap values with 1,000 replications (Felsenstein, 1985) were calculated by performing a heuristic search using the TBR option with MulTrees on.

In addition to MP analysis, maximum-likelihood (ML) and Bayesian analyses were also performed. For ML analysis, eight nested models of sequence evolution were tested for each data set using PhyML 3.0 (Guindon and Gascuel, 2003). For each data set,

the general time-reversible (GTR) (Lanave et al., 1984) substitution model led to a largest ML score compared to the other 7 substitution models: JC69 (Jukes and Cantor, 1969), K80 (Kimura, 1980), F81 (Felsenstein, 1981), F84 (Felsenstein, 1993), HKY85 (Hasegawa et al., 1985), TN93 (Tamura and Nei, 1993) and custom (data not shown). As a result, the GTR model was used in the Bayesian analysis using MrBayes 3.1 (Ronquist and Huelsenbeck, 2005). MrBayes 3.1 was run with the program's standard setting of two analyses in parallel, each with four chains, and an estimated convergence of results by calculating the standard deviation of split frequencies between analyses. In order to make the standard deviation of split frequencies fall below 0.01 so that the occurrence of convergence could be certain, 20,000 generations data were run for small subunit rRNA gene. Samples were taken every 1000 generations under the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites. For all analyses, the first 25% of samples from each run were discarded as burn-in to ensure the stationarity of the chains. Bayesian posterior probability (PP) values were obtained from a majority rule consensus tree generated from the remaining sampled trees.

A BLAST analysis was also conducted to determine if any of the microsporidia described from lady beetles were genetically similar to the microsporidium examined in this study; however, only *T. hippodamiae* from *H. convergens* had sequences submitted to GenBank.

1.3. Results

1.3.1 Pathogen ultrastructure

All stages of the microsporidium developed in direct contact with the host cell cytoplasm. Early developmental stages were observed adjacent to mature spores. Meronts were round (53/88 observations; 60.2%) to slightly irregular (35/88 observations; 39.7%,) and were surrounded by a thin plasma membrane (Fig. 1.1). Diplokaryotic nuclei were primarily observed (52/88 observations; 59.1%); however, meronts with a single nucleus were also observed (34/88 observations, 38.6%) and meronts with three nuclei were observed twice. Each nucleus was round and occupied roughly one-third of the cell. Numerous free ribosomes were within the cytoplasm but there was no evidence of an early developing endoplasmic reticulum. Merogony was not observed.

Round to oval sporonts (Fig. 1.2) were surrounded by a thickened, convoluted plasma membrane (17/27 observations; 63%). Free ribosomes were more abundant within the cytoplasm compared to the meront. The central area of the sporonts was occupied by a fused diplokaryon that was surrounded by the endoplasmic reticulum. Sporoblasts (Fig. 1.3) were highly irregular in shape and were surrounded by a thickened and convoluted plasma membrane. The diplokaryon was less apparent than it was in other vegetative stages. Sporoblasts contained a developing polar filament, a lamellar polaroplast and posterior vacuole.

Mature spores (Fig. 1.4) were diplokaryotic and measured 4.25 ± 0.09 x 1.82 ± 0.03 μm (± SE, n = 49, from micrographs). The polar filament was arranged in 10-18 coils (n = 42); however, several spores contained polar filaments that were arranged in 9

(*n* = 2), 20 (*n* = 1), 21 (*n* = 1) or 22 (*n* = 3) coils. The isofilar polar filament was frequently arranged in a single layer (37/49 observations; 75.5%) but double layers were also observed (12/49; 24.5%). The lamellar polaroplast (Fig. 1.5) was often not visible (14/49 observations) and the majority of spores contained a round membranous structure where the polaroplast is normally located (Fig. 1.6). The polar vacuole (Fig. 1.7) was frequently observed (31/49 observations; 63.3%) and electron-dense material was often observed along the internal perimeter (21/31; 67.7%). Spores were surrounded by a well-developed endospore and exospore (0.12 ± 0.004 μm and 0.03 ± 0.003 μm respectively, *n* = 49, from micrographs).

Numerous tubular-like structures surrounded by a delimiting membrane were observed within a single meront (Fig. 1.8) as well as in the central region of mature spores (4 observations, Fig. 1.9). Spherites (Fig. 1.10) were occasionally observed in proximity to microsporidian spores and vegetative stages. Evacuated (germinated) spores were frequently observed (Fig. 1.11).

1.3.2 Tissue Pathology

Spores were observed in the ovaries and developing oocytes in all four of the microsporidia-infected females examined. The spermatocytes and accessory glands within the testes of the four males examined were infected but the sperm bundles remained uninfected. In both sexes, the flight muscles and fat body were heavily infected and large numbers of spores were observed within and between the cells of these tissues. In the fat body, spores were frequently found within the cell nuclei. Spores were also

observed within the cells of the Malpighian tubules and in neurons that surround the ventral nerve cord. The midgut, ileum and colon epithelia were lightly infected and few microsporidian spores were observed in these tissues. Connective tissue beneath the cuticle and surrounding the trachea were also lightly infected.

1.3.3 Molecular characterization

Only one sequence was produced from microsporidia-infected *A. bipunctata* eggs, suggesting that only one species of microsporidia was present. Molecular analysis of the genome showed the pathogen described in this study was 97% similar to *Nosema bombi* (Accession no: AY008373.1) and 96% similar to *N. thomsoni* (Accession no: EU219086.1), *N. vespula* (Accession no: U11047.1), *N. oulemae* (Accession no: U27359.1), *Oligosporidium occidentalis* (Accession no: AF495379.1), *N. portugal* (Accession no: AF033316.1), *Vairimorpha lymantriae* (Accession no: AF033315.1) and *N. carpocapsae* (Accession no: AF426104.1; see Table 1.1). Maximum parsimony analysis using *N. apis* (Accession no: U26534.1) as the out group was conducted (25 parsimony-informative characters, 176 equally most parsimonious trees [CI = 0.801, RI = 0.733]). The separated Bayesian analyses using GTR model resulted in identical trees with mean log-likelihood values -2630.69 and -2641.84 (data not shown). The tree topologies were identical in both ML and Bayesian trees and similar to those generated by MP. Strict consensus trees with bootstrap (1000 replicates) and Bayesian PP values showed three different clades (Fig. 1.12). The microsporidium from *A. bipunctata* (*Nosema adaliae* n. sp.) formed one clade with *N. bombi* and *O. occidentalis* with 84%

bootstrap support (PP = 0.80). Within this clade, *N. bombi* and *O. occidentalis* formed a subclade with 83% bootstrap support (PP = 0.97). A BLAST analysis of the microsporidium from *A. bipunctata* and *T. hippodamiae* (described from *H. convergens*) revealed no significant similarities between the two sequences.

1.4. Discussion

Several microsporidia have been described from lady beetles; however, *N. coccinellae* is the only microsporidium known to naturally infect *A. bipunctata* (see Lipa et al., 1975). The description of *N. coccinellae* is based on light microscopic observations (pathogen life cycle, tissue pathology and spore dimensions) from *C. septempunctata*, *Hippodamia tredecimpunctata* (L.) and *Myrrha octodecimguttata* (L.). Although both *N. coccinellae* and the microsporidium in *A. bipunctata* belong to the genus *Nosema* and are described from lady beetles, spores of *N. coccinellae* (4.4-6.7 x 2.3-3.4 μm; Lipa, 1968, Lipa et al., 1975) are larger than those of the microsporidium from *A. bipuncata* (4.25 x 1.82 μm). However, spores of *N. coccinellae* were measured from fresh preparations whereas those reported in this study from *N. adaliae* were measured from TEM micrographs; the different methods of measurement could account for the difference in spore measurements.

The microsporidium *Tubulinosema hippodamiae*, described from the convergent lady beetle *H. convergens*, infects *A. bipunctata* under laboratory conditions. This pathogen is transmitted to *A. bipunctata, per os* when microsporidia-infected *H. convergens* eggs are eaten by uninfected *A. bipunctata* larvae (Saito and Bjørnson, 2008;

Steele and Bjørnson, 2012). Although the pathogen appears to have little observable effect on *A. bipunctata*, the two-spotted lady beetle appears to be a suitable host for *T. hippodamiae*. Spores are observed in *A. bipunctata* adults when they are fed microsporidia-infected eggs as first or second-instar larvae and the pathogen is transmitted vertically with 100% efficiency (Saito and Bjørnson, 2008; Steele and Bjørnson, 2012). The undescribed microsporidium from *A. bipunctata* shares some morphological characteristics with *T. hippodamiae* (diplokaryotic nuclei, all stages develop in direct contact with the host cell cytoplasm) but these two pathogens differ at the ultrastructural level. The most noticeable difference is that the polar filament of *T. hippodamiae* is anisofilar with 10 to 14 coils whereas the polar filament from the microsporidium in *A. bipunctata* is isofilar with 10 to 18 coils (Table 1.2). In addition, *T. hippodamiae* spores are smaller than those of the pathogen from *A. bipunctata* and the results of a BLAST analysis revealed that there were no similarities between the genomes of these two microsporidia. This latter suggests that these pathogens represent two distinct species.

According to the maximum identity score, the microsporidia that are most closely related to the microsporidium from *A. bipunctata* infect a wide range of different host species (Table 1.1). *N. bombi* was first described from the bumblebee *Bombus agrorum*. The description of *N. bombi* was originally based on smear preparations examined by light microscopy but recent descriptions now include ultrastructural examinations using TEM (see McIvor and Malone, 1995; Jilian et al., 2005). *N. thomsoni* is described from smear preparations of the large aspen tortrix moth, *Christoneura conflictana* (Wilson and

Burke, 1971). *N. vespula* is isolated from infected larvae of the European wasp, *Vespula germanica* but it is believed to infect a wide range of hosts, including hymenopterans, dipterans, and lepidopterans (Rice, 2001). The description of *N. vespula* is based solely on a partial sequence submission to GenBank. *N. oulemae* infects the cereal leaf beetle, *Ouelma melanopus* but the description of this pathogen is also based solely on a partial sequence submission to GenBank. The same applies to *V. lymantriae*, a microsporidium described from the gypsy moth, *Lymantria dispar*. *N. carpocapsae* infects the codling moth, *Cydia pomonella* (Malone and Wigley, 1981).

1.4.1 Pathogen ultrastructure

Molecular characterization of the microsporidium examined in this study revealed that it is 97% similar to *N. bombi* and 96% similar to several other species belonging to the genus *Nosema*, including *N. thomsoni*, *N. vespula*, *N. oulemae, N. portugal and N. carpocapsae*. Microsporidia belonging to the genus *Nosema* share several characteristics: all stages develop in direct contact with the host cell cytoplasm and diplokaryotic spores have an isofilar polar filament, a thin endospore with a moderately thick exospore and a distinctive, small polar vacuole (Sprague et al., 1992). The microsporidium from *A. bipunctata* also shared these characteristics. The microsporidium examined in this study is also 96% similar to *O. occidentalis* and *V. lymantriae*. The description of *V. lymantriae* is based solely on a genome submission to GenBank; however, there has been recent debate as to whether or not this microsporidium belongs to the genus *Nosema* or if *V. lymantriae* is an isolate of *V. disparis* (Vavra et al., 2006). Although *O. occidentalis* is

closely related to the pathogen from *A. bipunctata*, it lacks one of the characteristic features of the genus *Nosema*: a mature spore with a diplokaryotic nucleus.

Mature spores of the microsporidium from *A. bipuncata* and those of closely related microsporidia are similar in shape but differ with respect to size and the number of polar filament coils within (Table 1.3). Spores of the microsporidium from *A. bipunctata* measured 4.25 ± 0.09 x 1.82 ± 0.03 μm and are distinctively larger than those from the majority of closely related microsporidia, with exception of *N. bombi* and *N. portugal*. Compared to spores of *N. bombi* (4.88 ± 0.03 x 2.88 ± 0.03 μm; fresh), spores from *A. bipuncata* are slightly smaller but have fewer polar filament coils (10-18 coils from spores in *A. bipuncata* and 14-18 coils in *N. bombi*; Mclvor and Malone, 1995). Spores of *N. bombi* are also more-electron dense (appear darker) than those from *A. bipunctata* but this could be an artifact or it may reflect the maturity of the spores observed. Spores of *N. portugal* (4.5 ± 0.41 x 1.9 ± 0.17 μm, fresh) are similar in size to those from *A. bipuncata* but *N. portugal* also has fewer polar filament coils (10-11 coils in environmental spores; Maddox et al., 1999).

Ultrastructural studies have been completed for five of the eight species that are most closely related to the pathogen in *A. bipunctata* (including *N. bombi*, *N. thomsoni*, *O. occidentalis*, *N. portugal* and *N. carpocapsae*). The vegetative stages of the majority of these species are similar to those observed for the pathogen in *A. bipunctata*. *N. bombi* has slightly oval meronts with 1-4 nuclei and oval to fusiform sporonts with 1, 2 and 4 nuclei (McIvor and Malone, 1995). *N. thomsoni* has spherical meronts with 1-4 nuclei and elongated monokaryotic sporonts (light microscopic observations; Wilson and Burke,

1971). *N. portugal* has round to oval meronts with 2-4 nuclei and round diplokaryotic sporonts (Maddox et al., 1999) and *N. carpocapsae* has spherical meronts with 1, 2 or 4 nuclei and fusiform sporonts, also with 1, 2 or 4 nuclei (Malong and Wigley, 1981). Meronts of the microsporidium in *A. bipunctata* were spherical and primarily diplokaryotic (1 and 3 nuclei were also observed). Sporonts were round to oval with a fused diplokaryotic nuclei, which is typical for pathogens belonging to the genus *Nosema* (Sprague et al., 1992). *O. occidentalis* differs from the microsporidium examined in *A. bipunctata* because all vegetative stages and mature spores of *O. occidentalis* contain only a single nucleus (Becnel et al., 2002).

Tubular structures observed in spores from *A. bipunctata* were similar to those reported in *Nosema granulosis*. These are described as numerous discrete granules within the polaroplast at the anterior end of the spore (Terry et al., 1999). In the case of the microsporidium in *A. bipunctata*, the tubular structures were located within the central to posterior region of spores but did not occupy the polaroplast. Spherites, identified by their distinct concentric rings, were observed alongside spores and vegetative stages. These spherites were morphologically similar to those observed in the phytophagous lady beetle, *Epilachna cf. nylanderi* that are thought to assist the removal of excess minerals and toxins from the body (Rost-Roszkowska et al., 2010).

1.4.2 Tissue Pathology

Microsporidian spores were observed in various tissues including the midgut, hindgut (Malpighian tubules, ileum and colon), reproductive organs, fat body, ventral

nerve cord and flight muscles. The presence of spores in both the alimentary tract and ovaries (particularly within developing oocytes) suggests that the microsporidium can be transmitted *per os* (horizontally) and transovarially (vertically). In a previous study, *A. bipunctata* became infected with the undescribed microsporidium when uninfected, first-instar larvae were fed eggs from microsporidia-infected *A. bipunctata* adults (Steele and Bjørnson, 2012). Larvae that later eclosed as adults were used to determine the effects of the pathogen on the life history of adult beetles. All of the first-instar larvae that consumed a single microsporidia-infected egg became infected and vertical transmission of the pathogen was 100% after 30 days.

Other microsporidia found in lady beetles (*N. coccinellae, N. hippodamiae, T. hippodamiae and N. tracheophila*) infect various tissues in their respective hosts (Table 2). Of these, *N. coccinellae* is the only microsporidium reported from field-collected *A. bipunctata*. This pathogen infects the midgut epithelium, Malpighian tubules, gonads, nerves and muscle tissues of numerous beetles including *Coccinella septempunctata, Hippodamia tredecimpunctata and Myrrha octodecimguttata* (see Lipa, 1968). In the case of *A. bipunctata*, information regarding tissue pathology was not reported. The microsporidium, *T. hippodamiae* also infects *A. bipunctata* under laboratory conditions. The pathogen invades several tissues, including the fat body, muscles, Malpighian tubules, the pyloric valve, hindgut epithelium, ventral nerve cord, connective tissues and ovaries (see Saito, 2008). *N. hippodamiae* was found in the midgut epithelium, fat body and other unspecified tissues of *H. convergens* (see Lipa and Steinhaus, 1959). *N.*

tracheophila infects the tracheal epithelium, haemoctyes and connective tissues of *C. septempunctata* (see Cali and Briggs, 1967).

Based on the pathogen ultrastructure and molecular information gained during this study, we propose that the microsporidium in *A. bipunctata* be considered a new species and given the name *Nosema adaliae* sp. nov.

Taxonomic Summary (Nosema adaliae)

Nosema adaliae sp. nov. Steele and Bjørnson

GenBank Accession Number KC412706

Type host: *Adalia bipunctata* L. (Coleoptera: Coccinellidae)

Other hosts: unknown

Type locality: *A. bipunctata* field-collected in Nova Scotia, Canada.

Site of infection: Numerous tissues including the ovaries, developing oocytes, spermatocytes and accessory glands within the testes, midgut epithelium, Malpighian tubules, ileum, colon, fat body, ventral nerve cord and flight muscles.

Transmission: Horizontal transmission (*per os*) and evidence of vertical (transovarial) transmission (spores observed in ovaries and developing oocytes). Evacuated spores in host tissues provide evidence of autoinfection.

Merogony: Not observed

Sporogony: Not observed

Interface: All stages develop in direct contact with the host cell cytoplasm

Spores: Oval, diplokaryotic, measure 4.25 ± 0.09 x 1.82 ± 0.03 μm (± *SE*, *n* = 49, from micrographs). The isofilar polar filament was arranged in 10-18 coils in single and double layers. The lamellar polaroplast is often obscured by a round membranous structure and not visible. The relatively small polar vacuole is defined by electron-dense material along the internal perimeter.

Etymology: Specific name after the host genus.

Table 1.1. Species name, primary host, GenBank accession number and maximum identity score (MI) of ten microsporidian species closely related to the undescribed microsporidium from *Adalia bipuncata*.

Microsporidia	**Host**	**GenBank Accession #**	**MI (%)**
Nosema bombi	*Bombus agrorum* (Hymenoptera: Apidae)	AY008373.1	97
Nosema thomsoni	*Choristoneura conflictana* (Lepidoptera: Tortricidae)	EU219086.1	96
Nosema vespula	*Vespula germanica* (Hymenoptera: Vespidae)	EU219086.1	96
Nosema oulemae	*Oulema melanopus* (Coleoptera: Chrysomelidae)	U27359.1	96
Oligosporidium occidentalis	*Metaseiulus occidentalis* (Mesostigmata: Phytoseiidae)	AF495379.1	96
Nosema portugal	*Lymantria dispar* (Lepidoptera: Erebidae)	AF033316.1	96
Vairimorpha lymantriae	*Lymantria dispar* (Lepidoptera: Erebidae)	AF033315.1	96
Nosema carpocapsae	*Cydia pomonella* (Lepidoptera: Tortricidae)	AF426104.1	96
Vairimorpha necatrix	*Pseudaletia unipuncta* (Lepidoptera: Noctuidae)	DQ996241.1	95
Nosema necatrix	*Pseudaletia unipuncta* (Lepidoptera: Noctuidae)	U11051.1	95

Table 1.2. Histological and ultrastructural comparison of microsporidian pathogens described from lady beetles.

Microsporidia	Host(s)	Tissues Infected	Spore Shape	Spore Size	Nuclei (#)	PF Coils	PF Type	Reference
Nosema hippodamiae	*Hippodamia convergens*	Mg, FB	Ovoid	3.3 - 5.4 x 2.2 - 2.7 μm[1]	1	N/A	N/A	Lipa & Steinhaus, 1959
Nosema tracheophila	*Coccinella septempunctata*	TE, He, CT	Ovoid	4.0 - 5.3 x 2.2 - 3.1 μm[2]	N/A	N/A	N/A	Cali & Briggs, 1967
Nosema coccinellae	*Coccinella septempunctata, Hippodamia tredecimpunctata, Myrrha octodecimguttata, Adalia bipunctata*	ME, MT, G, N, M	Elipsoidal	4.4 - 6.7 x 2.3 - 3.4 μm[2]	N/A	N/A	N/A	Lipa, 1968; Lipa et al., 1975
Tubulinosema hippodamiae	*Hippodamia convergens*	FB, M, MT, PV, Hg, VN, CT, Ov	Pyriform	3.58 ± 0.2 x 2.06 ± 0.2 μm[3]	2	10 - 14	Anisofilar	Bjørnson et al., 2011
Nosema adaliae	*Adalia bipunctata*	Ov, T, ME, MT, Hg, FB, VN, M	Ovoid	4.25 ± 0.09 x 1.82 ± 0.03 μm[3]	2	10 - 18	Isofilar	

CT = connective tissues; FB = fat body; G = gonads; He = haemocytes; Hg = hindgut; MT = Malpighian tubules; Mg = midgut; ME = midgut epithelium; M = muscle; N = nerves; Ov = ovaries; PF = polar filament; PV = pyloric valve; T = testes; TE = tracheal epithelium; VN = ventral nerve cord.
N/A: Information is lacking from the formal description
[1] Measurements taken from an alcohol fixed & Giemsa stained smear
[2] Measurements taken from fresh smear
[3] Measurements based off of micrographs

Table 1.3. Ultrastructural comparison of eight microsporidian species most closely related to the microsporidium from *Adalia bipuncata* with a maximum identity score of 95% or higher. Shape refers to spore shape; PF = polar filament; PP = polaroplast.

Microsporidia	Host	Shape	Spore Size	Nuclei	PF Coils	PF Type	PP Type	Reference
Nosema bombi	*Bombus agrorum* (Hymenoptera: Apidae)	Ovoid	4.88 ± 0.03 x 2.88 ± 0.03 mm[1]	2	14-18	Isofilar	Lamellar	McIvor & Malone, 1995
Nosema thomsoni	*Choristoneura conflictana* (Lepidoptera: Tortricidae)	Ovoid	1.1 - 1.8 x 2.1 - 3.1 mm [1]	1[4]	N/A	N/A	N/A	Wilson & Burke, 1971
Nosema vespula	*Vespula germanica* (Hymenoptera: Vespidae)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nosema oulemae	*Oulema melanopus* (Coleoptera: Chrysomelidae)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Oligosporidium occidentalis	*Metaseiulus occidentalis* (Mesostigmata: Phytoseiidae)	Oblong-ovoid	2.53 ± 0.06 x 1.86 ± 0.06 μm[1]	1	3 - 5, 8 - 9 [5]	Isofilar	Bipartite lamellar	Becnel et al., 2002
Nosema portugal	*Lymantria dispar* (Lepidoptera: Erebidae)	Ovoid	4.5 ± 0.41 x 1.9 ±0.17 μm[1]	2	5 - 8, 10 - 11[5]	Isofilar	Lamellar	Maddox et al., 1999
Vairimorpha lymantriae	*Lymantria dispar* (Lepidoptera: Erebidae)	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Nosema carpocapsae	*Cydia pomonella* (Lepidoptera: Tortricidae)	Ovoid	2.4 - 3.9 × 1.3 - 3.1 μm[2]	2	9-13	N/A	N/A	Malone & Wigley, 1981
Nosema adaliae	*Adalia bipunctata* (Coleoptera: Coccinellidae)	Ovoid	4.25 ± 0.09 x 1.82 ± 0.03 μm[3]	2	10-18	Isofilar	Lamellar	

N/A = information is not available; pathogen is either undescribed or information is lacking from description
[1] Measurements taken from fresh smear preparations
[2] Measurements taken from fixed & stained (Geimsa) preparations
[3] Measurements taken from micrographs
[4] Information based on light microscopic observations
[5] Primary spore and environmental spore; respectively

Figures 1.1 to 1.7: Ultrastructural characteristics of the life cycle of Nosema adaliae sp. nov., from the two-spotted lady beetle, Adalia bipunctata.

Fig. 1.1. Diplokaryotic meront showing two distinct nuclei (N) and free-floating ribosomes surrounded by a thin plasma membrane.

Fig. 1.2. Sporont with two nuclei (N) and thickened plasma membrane (arrow). Endoplasmic reticula (ER) are visible around the perimeter of the nuclei.

Fig. 1.3. Early-stage diplokaryotic sporoblast with two nuclei (N), a developing polar filament (PF) and lamellar polaroplast (PP) surrounded by a thickened, convoluted plasma membrane.

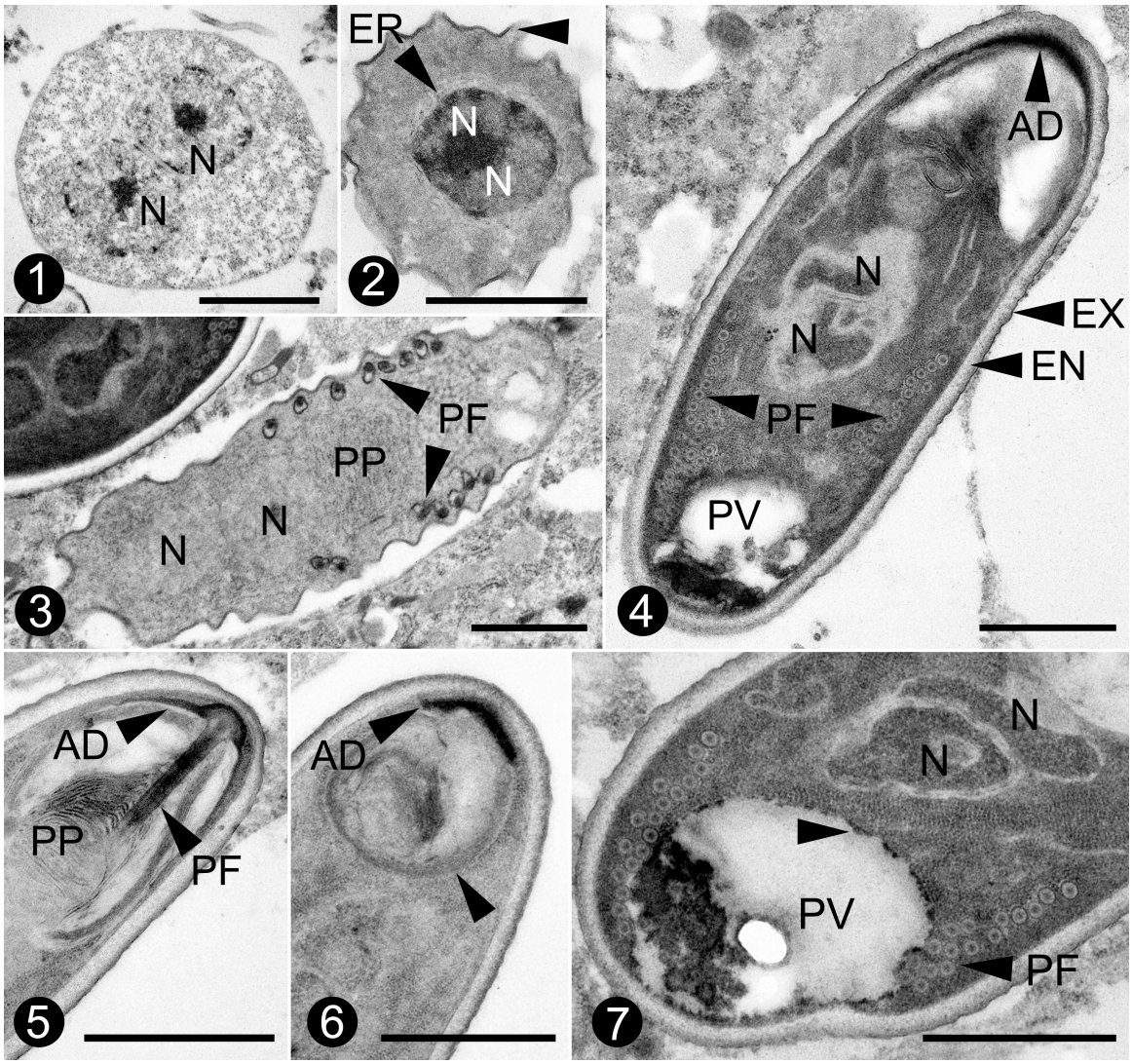
Fig. 1.4. Mature spore with a fully developed spore wall, composed of an exospore (EX) and endospore (EN), a diplokaryotic nucleus (N) and an isofilar polar filament (PF) arranged in a double layer. A polar vacuole (PV) is visible within the posterior region of the spore and part of anchoring disk (AD) at the anterior end.

Fig. 1.5. The anterior region of a mature spore showing details of the anchoring disk (AD), polar filament (PF) and lamellar polaroplast (PP).

Fig. 1.6. Apical region of a mature spore showing a portion of the anchoring disk (AD) and a round membranous structure where the polaroplast is normally located (arrow).

Fig. 1.7. Posterior region of a mature spore showing the polar vacuole (PV) with electron-dense material along the internal perimeter (arrow), polar filament (PF) coils arranged in a double row, and a clearly defined diplokaryotic nucleus (N).

Scale bars: 1 μm.


1
N
N
2
ER
N
N
3
PF
PP
N
N
4
AD
N
N
EX
EN
PF
PV
5
AD
PP
PF
6
AD
7
N
N
PV
PF

Figures 1.8 to 1.11. Anomalous structures and germinated spores.

Fig. 1.8. Meront with tubular structures (arrow) of unknown origin or significance delimited by a membrane.

Fig. 1.9. Numerous tubular structures within the central region of mature spores.

Fig. 1.10. Spherite (arrow) adjacent to a mature spore (SP).

Fig. 1.11. Evacuated (germinated) spores provide evidence that autoinfection occurs.

Scale bars: Figs. 8 and 9, 1 μm; Fig. 10, 0.5 μm; Fig. 11, 2 μm.

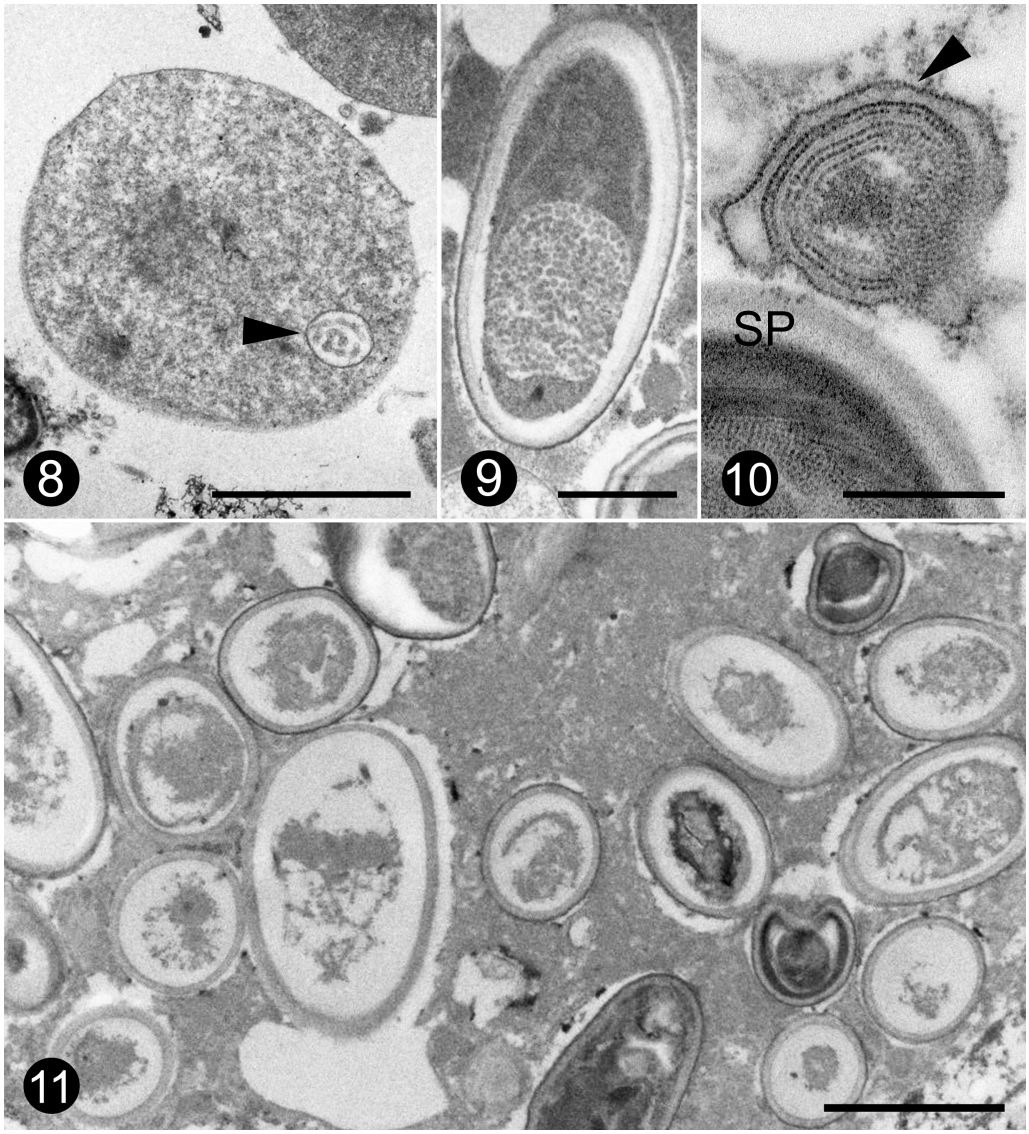
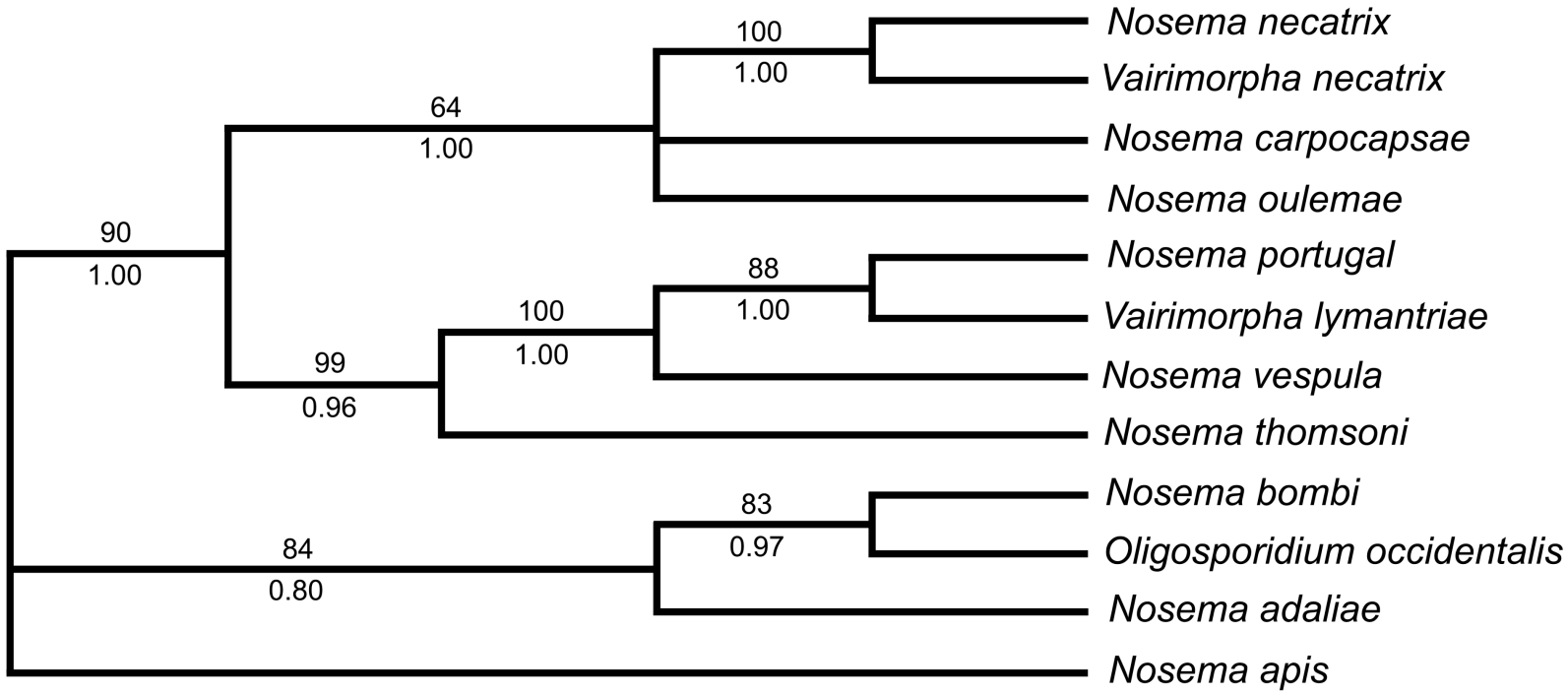

8
9
SP
10
11

Fig. 1.12. Strict consensus tree derived from 16S ribosomal RNA gene sequence data conducted using a heuristic search with TBR branch swapping. Numbers above and below branches are bootstrap and Bayesian posterior probability (PP) values, respectively. *Nosema apis* was used as an out group. Consistency index (CI) = 0.801, retention index (RI) = 0.733.


100
1.00
Nosema necatrix
Vairimorpha necatrix
64
1.00
Nosema carpocapsae
Nosema oulemae
90
1.00
Nosema portugal
88
1.00
100
1.00
Vairimorpha lymantriae
99
0.96
Nosema vespula
Nosema thomsoni
Nosema bombi
83
0.97
84
0.80
Oligosporidium occidentalis
Nosema adaliae
Nosema apis

Chapter Two

Ultrastructure and molecular characterization of the microsporidium, *Nosema chrysoperlae* sp. nov., from the green lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae) used for biological pest control

2.1 Introduction

During a trip to Australia in 1888, Albert Koebele was first to observe the predaceous behavior of lacewing larvae as they fed on the cottony cushion scale, *Icerya purchasi* Maskell (Koebele, 1890). The biological control potential of lacewings was soon fully appreciated and mass-culturing techniques for inundative release were developed (Finney, 1950). Today, both *Chrysoperla carnea* (Stephens) and *C. rufilabris* (Burmeister) are mass-produced for aphid control on various crops in Europe and North America (van Lenteren et al. 1997).

Lacewings are well known for their symbiotic association with yeasts, bacteria and filamentous fungi. Yeasts of the genera *Candida* and *Metschnikowia* are commonly reported in lacewings (Suh et al. 2004; Woolfolk and Inglis, 2004; Nguyen et al. 2006) and although the specific role of yeasts is poorly understood, they are thought to provide the host with a source of nutrients. More than 25 taxa of bacteria have been isolated from the alimentary canal of adult *C. rufilabris* and both larval and adult *C. carnea*. Dense populations of these microbes in the midgut of *C. carnea* larvae suggests that bacteria are involved in the decomposition of food or are otherwise beneficial for larval growth and development (Chen et al. 2006). Filamentous fungi from six taxa have also been isolated from *C. rufilabris*. The infrequent presence of bacteria and filamentous fungi in the

alimentary canal of chrysopids suggests that these microbes are transient and are unlikely to form symbiotic relationships with lacewings (Woolfolk and Inglis, 2004).

There is only one report of an infection caused by microsporidia in lacewings. In 1949, the microsporidium *Plistophora californica* Steinhaus & Hughes, a pathogen of the potato tuberworm, *Gnorimoschema operculella* (Zeller) was noted to infect several other insect hosts, including *Chrysoperla californica* Coquillet (Steinhaus and Hughes, 1949). At that time, the potato tuberworm was a significant pest when rearing mealybugs as food for the mass-production of the predatory coccinellid, *Cryptolaemus montrouzieri* Mulsant (see Finney et al. 1947). Although *C. californica* is a non-target host of *P. californica*, infection reduces lacewing longevity and fecundity (Finney, 1950).

In this study, we describe a microsporidium from *Chrysoperla carnea* that was detected during the examination of specimens obtained from a commercial insectary for biological pest control. This is the first report and formal description of a microsporidium from *C. carnea*. The objective of this study was to describe the pathogen by means of its ultrastructure, molecular characterization and tissue pathology.

2.2 Materials and Methods

In 2010, *C. carnea* larvae were purchased from a European commercial insectary for use in a laboratory study. During the rearing process, some larvae turned black and died in their second- or third-instar. A microsporidian pathogen was detected in smear preparations of these individuals when examined by light microscopy.

In an attempt to study the transmission and effects of the pathogen, additional *C. carnea* larvae were reared in 47 mm-diameter Petri dishes (Millipore) under controlled

conditions (16:8 L:D; 25°C:20°C). Each dish had a 2.2 cm hole cut in the lid that was covered with a fine mesh screen (80 microns), allowing air circulation. One larva was reared per dish. Larvae were fed green peach aphids (*Myzus persicae* Sulzer) and distilled water was provided through a moistened cotton wick (Crosstex International, NY). Individuals from aphid colonies were screened for microsporidia when the laboratory colony was established and on a routine basis during the experimental trials. Larvae were reared to adult and upon eclosion, some adults exhibited malformed wings that had a characteristic 'clubbed' appearance. Both asymptomatic and symptomatic adult specimens were processed for histological and microscopic examination. Specimens were also sent for sequencing to determine the molecular characterization of the pathogen. Attempts to rear microsporidia-infected *C. carnea* for further study were unsuccessful and vertical transmission was not confirmed.

2.2.1 Pathogen ultrastructure

Adult specimens (n = 23) reared individually from symptomatic mothers (those with 'clubbed' wings) were embedded for examination by transmission electron microscopy (TEM). Only two of these individuals were confirmed to be infected with the microsporidium and both were examined by TEM. Adult *C. carnea* were submerged and dissected in 2.5% glutaraldehyde. The head, wings and legs were removed from each specimen to encourage infiltration of the fixative. The thorax of each specimen was removed from its abdomen and each was processed separately. Tissues were stored in fixative (4°C; 48-72 h) until they were processed according to the procedure by Becnel (1997, see Appendix A) with the following modification: tissues were placed under

vacuum (15 psi) overnight once they were in pure resin. Tissues were embedded in Jembed 812/Spurr resin (1:1, Canemco & Marivac, QC). Specimens were processed in two batches.

Ultra-thin (70 nm) sections cut with a Leica UCT ultramicrotome were stained with uranyl acetate and lead citrate prior to examination. Digital micrographs were generated with a GATAN ES500W Erlangshen CCD camera side mounted to a Hitachi H7500 transmission electron microscope at 80 kV. Imaging software (ImageJ) was used for determining spore measurements.

2.2.2 Tissue pathology

Fifteen adult *C. carnea* were embedded for histological examination and a total of 10 were examined (4 uninfected and 6 microsporidia-infected). Each specimen was submerged and dissected in Carnoy's fixative (60 ml ethanol, 30 ml chloroform, 10 ml glacial acetic acid). The head, wings and legs were removed from each individual but the thorax was left attached to the abdomen. Prior to processing, a small piece of tissue excised from anterior region of the prothorax was examined to confirm infection by light microscopy.

Specimens were stored in fixative (4°C; 48-72 h) until they were processed according to the procedure by Becnel (1997, see Appendix B), with the following modifications: (1) tissues remained submerged in 1:1 ethanol:butanol overnight in a >25.5°C oven (instead of 2h at room temp); (2) tissues were submerged in 3:1 butanol:paraffin for 20 min in a 60°C oven (this step was inserted between 100% butanol and 1:1 butanol:paraffin); and (3) tissues remained in 1:1 butanol paraffin overnight (instead of 2 h). Tissues were

embedded in Paraplast® Plus (Sigma-Aldrich Inc., melting point 56°C). Specimens (both uninfected and infected) were processed simultaneously in three batches.

Serial, longitudinal sections (5 µm thickness) cut with a rotary microtome were fixed to slides that had been pre-coated with a protein solution (1 g gelatin, 2 g solid phenol, 15 ml glycerin, 100 ml distilled water) to enhance fixation. Once dry, the paraffin was removed with xylene and sections were rehydrated with solutions of decreasing ethanol concentrations until 100% distilled water was achieved. Tissues were stained with Harris hematoxylin and alcoholic Eosin Y (Fisher Scientific, see Appendix C) and examined by light microscopy.

2.2.3 Molecular characterization

Microsporidia genomic DNA was extracted from *C. carnea* adults according to the methods described by Bjørnson et al. (2011). DNA was isolated and purified with a DNeasy® Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. Microsporidian primers described by Vossbrinck et al. (2004) were used to design primers for this study (18f (CACCAGGTT GATTCT GCC)/1492 (GGTTACCTTGTTACGACTT); Eurofins Laboratories). DNA was amplified by Polymerase Chain Reaction (PCR), cloned using pGEM®-T and pGEM®-T Easy Vector Systems (Promega Corp.), extracted with PureYield™ Plasmid Miniprep System (Promega Corp.), and sent to Macrogen (Korea) for sequencing.

Sequences of *Nosema bombycis*, *N. furnacalis*, *N. granulosis* and *N. spodopterae* were obtained from NCBI GeneBank. *N. apis* was used for comparison as an outgroup. Automated sequence outputs were visually inspected with chromatographs. Multiple

sequence alignments were made using ClustalX with default parameters and additional manual edits to minimize gaps (Thompson et al. 1997). Phylogenetic analysis using the maximum-parsimony (MP) method was performed with the computer program PAUP* ver. 4 beta 10 (Swofford, 2003). All characters were specified as unweighted and unordered, and gap-only columns were excluded in the analyses. The most parsimonious trees were constructed by performing a heuristic search using Tree Bisection-Reconnection (TBR) with the following parameters: MulTrees on and 10 replications of random addition sequences with the stepwise addition option. Multiple parsimonious trees were combined to form a strict consensus tree. Overall character congruence was estimated by the consistency index (CI) and the retention index (RI). To infer the robustness of clades, bootstrap values with 1,000 replications (Felsenstein, 1985) were calculated by performing a heuristic search using the TBR option with Multree on.

In addition to MP analysis, maximum-likelihood (ML) and Bayesian analyses were also performed. For the ML analysis, eight nested models of sequence evolution were tested for each data set using PhyML 3.0 (Guindon and Gascuel, 2003). For each data set, the general time-reversible (GTR) (Lanave et al. 1984) substitution model led to a largest ML score compared to the other 7 substitution models: JC69 (Jukes and Cantor, 1969), K80 (Kimura, 1980), F81 (Felsenstein, 1981), F84 (Felsenstein, 1993), HKY85 (Hasegawa et al. 1985), TN93 (Tamura and Nei, 1993) and custom (data not shown). As a result, the GTR model was used in the Bayesian analysis using MrBayes 3.1 (Ronquist and Huelsenbeck, 2005). MrBayes 3.1 was run with the program's standard setting of two analyses in parallel, each with four chains, and an estimated convergence of results was determined by calculating the standard deviation of split frequencies between analyses. In

order to make the standard deviation of split frequencies fall below 0.01, so that the occurrence of convergence could be certain, 110,000 generations data were run for small subunit rRNA gene. Samples were taken every 1000 generations under the GTR model with gamma-distributed rate variation across sites and a proportion of invariable sites. For all analyses, the first 25% of samples from each run were discarded as burn-in to ensure the stationarity of the chains. Bayesian posterior probability (PP) values were obtained from a majority rule consensus tree generated from the remaining sampled trees.

2.3. Results

Microsporidia were not detected in the aphids that were examined from the colonies used as food for *C. carnea*. Some *C. carnea* larvae that were reared in the lab turned from mottled ivory and reddish-brown (Fig. 2.1a) to black (Fig. 2.1b). These specimens died prematurely, often late in their second- or third-instar. Smear preparations of these larvae revealed microsporidian spores. Some of the microsporidia-infected larvae did not turn black and these individuals developed successfully to eclose as adults. Uninfected green lacewing adults have characteristic, long and delicate wings with network venation (Fig 2.1c). In contrast, microsporidia-infected adults were often identified by their distinctive, malformed wings that had a characteristic ‘clubbed’ appearance (Figs. 2.1 d, e). In such cases, the wings appeared to be fused into a mass of solid tissue. Some of these infected adults were unable to eclose successfully and died with remnants of larval exuvia attached to their bodies or were otherwise unable to emerge completely from their pupal case.

2.3.1 Pathogen ultrastructure

All stages of the microsporidium developed in direct contact with the host cell cytoplasm. Round to oval meronts (Fig. 2.2) contained a cytoplasm with free ribosomes that was surrounded by a thin, plasma membrane. Diplokaryotic nuclei were primarily observed (80/152 observations; 52.6%). However, meronts with one, three or four nuclei were observed (66/152 observations, 43.4%; 4/152, 2.6%; and 2/152, 1.3% respectively) but it is likely that these observations were sectioning artifacts or represent nuclei that are undergoing division. The nuclei of meronts occupied two thirds of the cell and there was evidence of an early-developing endoplasmic reticulum around the nuclear perimeter. Merogony was not observed.

Sporonts (Fig. 2.3) were oval to irregular in shape. The diplokaryon occupied a large, central region of the cell and was surrounded by a well-developed endoplasmic reticulum. Condensed chromatin was often visible within the nuclei. The thickened plasma membrane was often undulated in appearance and the cytoplasm contained more free ribosomes than did the meront. Tubular structures were observed in sporonts (24/61 observations, 39.3%; Fig. 2.4) as well as in spores (7/37, 18.9%).

Sporoblasts (Fig. 2.5) were highly irregular in shape. They were surrounded by a thickened, somewhat convoluted plasma membrane and contained a diplokaryon. The cytoplasm was filled with free ribosomes and contained endoplasmic reticulum and a developing polar filament and anchoring disc. Sporogony was not observed.

Diplokaryotic spores (Fig. 2.6) measured 3.49 ± 0.10 x 1.52 ± 0.05 μm (± SE; n = 37, from micrographs). The isofilar polar filament was arranged in 8-10 coils (n = 34) with three exceptions (when 5, 6, or 11 coils were observed). The polar filament was

frequently arranged in a single row (29/37 observations; 78.4%) but double rows were also observed (8/37; 21.6%). There was evidence of polar filament extrusion within the host tissues (Fig. 2.7) and evacuated (germinated) spores were observed (data not shown). Mature spores had a lamellar polaroplast (Fig. 2.8) and an anchoring disc with relatively long arms. A relatively small and inconspicuous polar vacuole was observed in about half of the spores that were examined (19/37 observations). Spores were surrounded by a well-developed spore wall and a cluster of small tubules was often observed in the posterior region of the spore (18/37 observations, 46.7%; Fig. 2.9).

2.3.2 Tissue Pathology

Four of the six microsporidia-infected specimens examined were female. The microsporidium infected several tissues of both male and female specimens. Microsporidian spores were observed in cells of the proventriculus and diverticulum (foregut, not shown). When intact, the epithelial cells in the anterior region of the midgut were uninfected whereas those in the posterior region contained few microsporidian spores. In four of six cases, the midgut epithelial cells were damaged severely as a result of infection. Damaged cells did not have any microvilli and often only small remnants of these cells remained. The Malpighian tubules, ilium, and rectum (hindgut; Fig. 2.10) were heavily infected and large numbers of microsporidian spores filled these cells.

The fat body and peripheral region of the thoracic and abdominal ganglia (Fig. 2.11) were also infected and spores occupied regions within and between the flight muscles (Fig. 2.12). Spores were observed beneath the cuticle and in one case they were observed in the tissue surrounding the trachea (Fig. 2.13). Although the tissues adjacent to

the ovaries were heavily infected, microsporidian spores were not observed within the developing eggs in any of the females examined. Spores were observed in the gonads of one of the two males examined.

2.3.3 Molecular characterization

Molecular analysis of the genome showed the pathogen described in this study was 99% similar to *N. bombycis* (Accession no. AY209011.1), *N. furnacalis* (Accession no: U26532.1), *N. granulosis* (Accession no: AJ011833.1) and *N. spodopterae* (Accession no: AY747307.1). Maximum parsimony analysis using *N. apis* as the outgroup was conducted (26 parsimony-informative characters, 483 equally most parsimonious trees (CI = 0.981, RI = 0.743). The separated Bayesian analyses using GTR model resulted in identical trees with mean log-likelihood values -3008.69 and -3013.82 (data not shown). The tree topologies were identical in both ML and Bayesian trees and similar to those generated by MP. Strict consensus trees with bootstrap (1000 replicates) value and Bayesian PP showed two different clades (Fig. 2.14). The microsporidium described in this study formed one clade with *N. furnacalis* and *N. granulosis* with 74% bootstrap support. Within this clade, *N. furnacalis* and *N. granulosis* formed a subclade. *N. bombycis* and *N. spodopterae* formed a separate clade (BS = 98%, PP = 0.96).

2.4. Discussion

Microsporidia are common pathogens of natural enemies, including those that are mass-produced and commercially available for biological pest control (Bjørnson and Schütte, 2003). Microsporidia have been reported in the predatory mites *Phytoseiulus*

persimilis Athias-Henriot (Bjørnson et al. 1996; Bjørnson, and Keddie, 2000), *Neoseiulus cucumeris* Oudemans and *N. barkeri* Hughes (Beerling and van der Geest, 1991), *Metaseiulus occidentalis* (Nesbitt) (Becnel et al. 2002) as well as the pteromalid endoparasitoid *Muscidifurax raptor* Girault and Saunders (Geden et al. 1992). Microsporidia are also pathogens of field-collected convergent lady beetles, *Hippodamia convergens* Guérin-Méneville (Bjørnson et al. 2011) that are redistributed for aphid control. The microsporidium described from *C. carnea* is yet another example of a cryptic pathogen from a mass-produced natural enemy that may have otherwise gone unnoticed if not for the routine examination of individuals for pathogens.

Three of the four microsporidia that are most closely related to the microsporidium found in *C. carnea* infect lepidopteran hosts. *N. bombycis* is perhaps best known for causing pébrine disease in silkworms, *Bombyx mori* L. (Tanada and Kaya, 1993) but this pathogen also infects other lepidopterans, most notably the small and large white, *Pieris rapae* L. and *P. brassicae* L., respectively (Kashkarova and Khakhanov, 1980). *N. furnacalis* was initially described from the Asian corn borer, *Ostrinia furnacalis* (Guenée) (see Oien and Ragsdale, 1993) and *N. spodopterae* is a pathogen of the tobacco cutworm *Spodoptera litura* Fabr. (see Johny et al., 2005). In contrast, *N. granulosis* is the only pathogen that is closely related to the microsporidium in *C. carnea* to infect an amphipod, *Gammarus duebeni* Liljeborg (see Terry et al., 1999).

Some infected *C. carnea* larvae and adults showed distinct signs associated with infection. Some larvae turned black during the latter part of their development and died but others were able to complete development and eclose successfully as adults. Many of these infected adults had deformed wings that had a distinct ‘clubbed’ appearance. In

contrast, the microsporidium *Plistophora californica* infects both the larvae of the potato tuber worm (*G. operculella*) and *C. californica*. Heavily infected *G. operculella* larvae are somewhat opaque and whitish in appearance; however, there is no mention of any signs associated with infection of *C. californica* (Steinhaus and Hughes, 1949).

2.4.1 Pathogen ultrastructure

Spores of the microsporidium in *C. carnea* measured 3.49±0.10 x 1.52±0.05 μm and were larger than those of *P. californica* (1.5-3.0 x 0.8-1.2 μm; mean dimensions: 2.0 x 1.0 μm; Steinhaus and Hughes, 1949). Spores in *C. carnea* had a relatively small polar vacuole, a characteristic of *Nosema* sp. (Sprague et al. 1992) and all stages of the pathogen were diplokaryotic and developed in direct contact with the host cell cytoplasm. Vegetative stages and spores contained tubular structures; those in the vegetative stages were similar in appearance to those in *Nosema granulosis* (see Terry et al. 1999).

In the case of *P. californica*, sporonts have one to four nuclei and in most cases, they develop into multi-nucleated, plasmodia-like structures (pansporoblasts) that contain several nuclei. Each nucleus develops into a mature spore. Steinhaus and Hughes (1949) provide diagrams of *P. californica* development and although the number of nuclei per spore is not stated, the diagrams depict one nucleus per spore. Individual *P. californica* spores or groups of spores (most with 16 spores; range: 8 to >100 spores per group) are observed in fresh specimens. Although merogony and sporogony were not observed in the microsporidium from *C. carnea*, there was no evidence of plasmodia-like structures similar to what was described for *P. californica*.

2.4.2 Tissue Pathology

Microsporidian spores were observed in the foregut (proventriculus and diverticulum), the posterior region of the midgut, and the hindgut (Malpighian tubules, ilium, and rectum) . As a result of infection, the midgut epithelial cells often lacked microvilli and few cells remained intact. The presence of spores in the alimentary tract, particularly in the Malpighian tubules and rectum, supports the assumption that this pathogen is transmitted *per os*. Horizontal transmission may occur though several mechanisms but because cannibalism is common among lacewing larvae, the pathogen is likely transmitted when infected larvae or infected adults are eaten by uninfected larvae. Although the pathogen from *C. carne*a is closely related to *N. furnacalis*, the latter does not infect *C. carnea* larvae when they are fed spore solutions under laboratory conditions (Oien and Ragsdale, 1993). Polar filament extrusion (Fig. 2.7) and evacuated (germinated) spores within *C. carnea* tissues provide evidence that autoinfection does occur.

Based on the information on pathogen ultrastructure and the molecular information gained during this study, we propose that this previously undescribed species of microsporidia in *C. carnea* be considered a new species and given the name *Nosema chrysoperlae* sp. nov.

***Taxonomic Summary* (*Nosema chrysoperlae*)**

Nosema chrysoperlae sp. nov., Bjørnson, Steele, Hu, Ellis & Saito

GenBank Accession Number KC412707

Type host: *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae)

Other hosts: unknown

Type locality: *C. carnea* purchased from a European commercial insectary for biological pest control.

Site of infection: Malpighian tubules, ileum, and rectum (hindgut) are heavily infected. Other infected tissues include the proventriculus, diverticulum, epithelial cells (posterior midgut), fat body, ganglia, and flight muscles. Spores were observed in the gonads of one of two males examined but not within developing eggs.

Transmission: Autoinfection was observed. Infection of the alimentary canal suggests that horizontal transmission occurs *per os*. There is no evidence to confirm vertical transmission.

Merogony: Not observed.

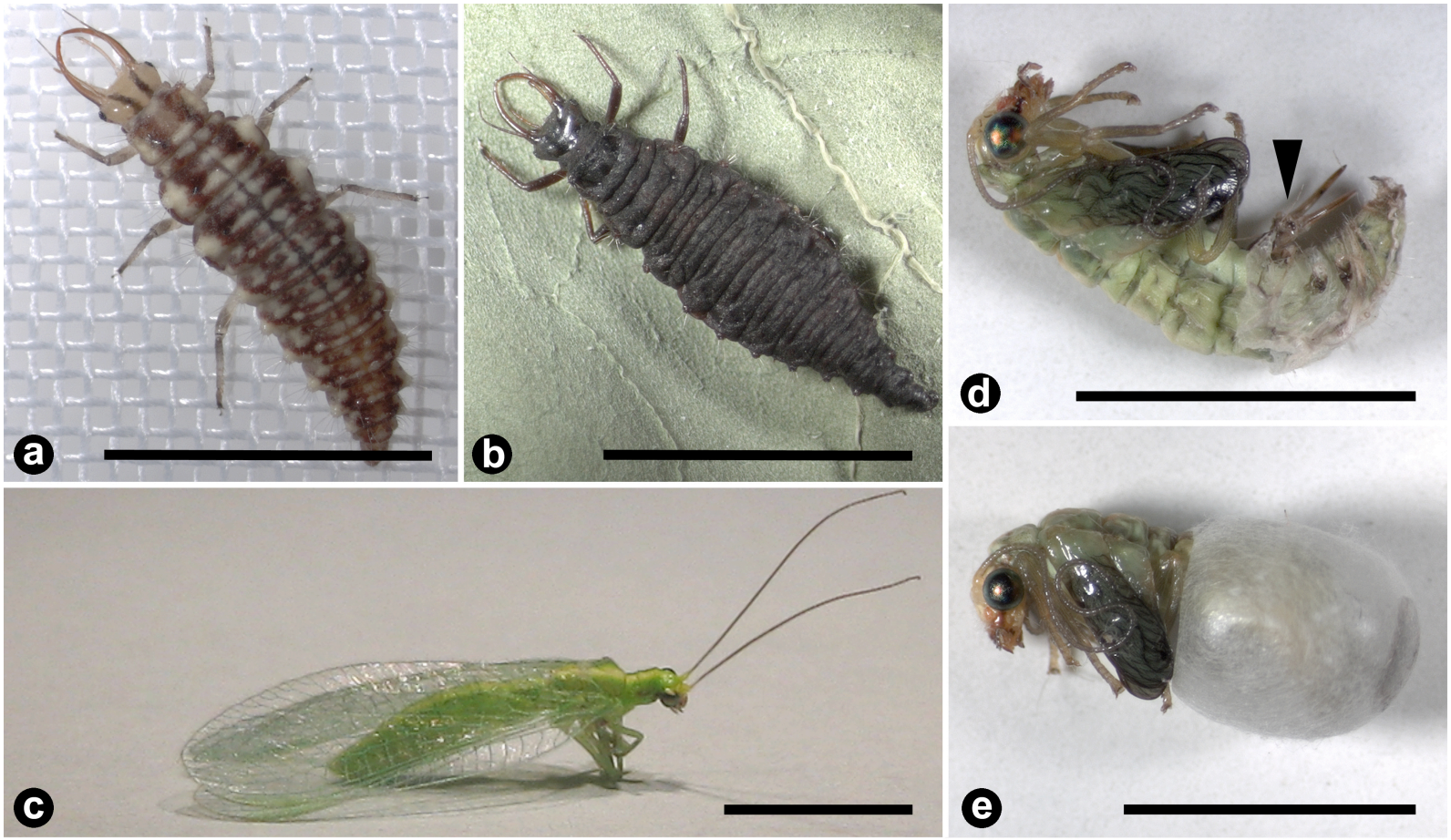
Sporogony: Not observed.

Interface: All stages of the microsporidium developed in direct contact with the host cell cytoplasm.

Spores: Diplokaryotic, 3.49 ± 0.10 x 1.52 ± 0.05 μm (n = 37, from micrographs) with a lamellar polaroplast, relatively inconspicuous polar vacuole and a polar filament arranged in 8-10 coils (n = 34) in a single (or occasionally double) row. Clusters of tubular structures were observed in both vegetative stages and spores.

Etymology: Specific name refers to the host genus *Chrysoperla*, from which the pathogen was described.

Fig. 2.1. *Chrysoperla carnea* third-instar larvae and adults: (a) Live, uninfected larva; (b) Dead, microsporidia-infected larva that turned black as a result of infection; (c) Live, uninfected *C. carnea* adult; (d) Dead, partially eclosed, microsporidia-infected adult with 'clubbed' wings. The pupal case is lacking but the larval exuvia, including the larval head capsule (arrow) remains attached; (e) Dead adult with deformed, 'clubbed' wings that was unable to eclose from its pupal case. Scale bars: 0.5 mm.


a
b
c
d
e

Figures 2.2 to 2.6. Vegetative stages and mature spores of Nosema chrysoperlae sp. nov.

Fig. 2.2. Diplokaryotic meront in direct contact with the host cell cytoplasm. The meront cytoplasm contains free ribosomes, a developing endoplasmic reticulum (ER), two nuclei (N) and is surrounded by a thin plasma membrane.

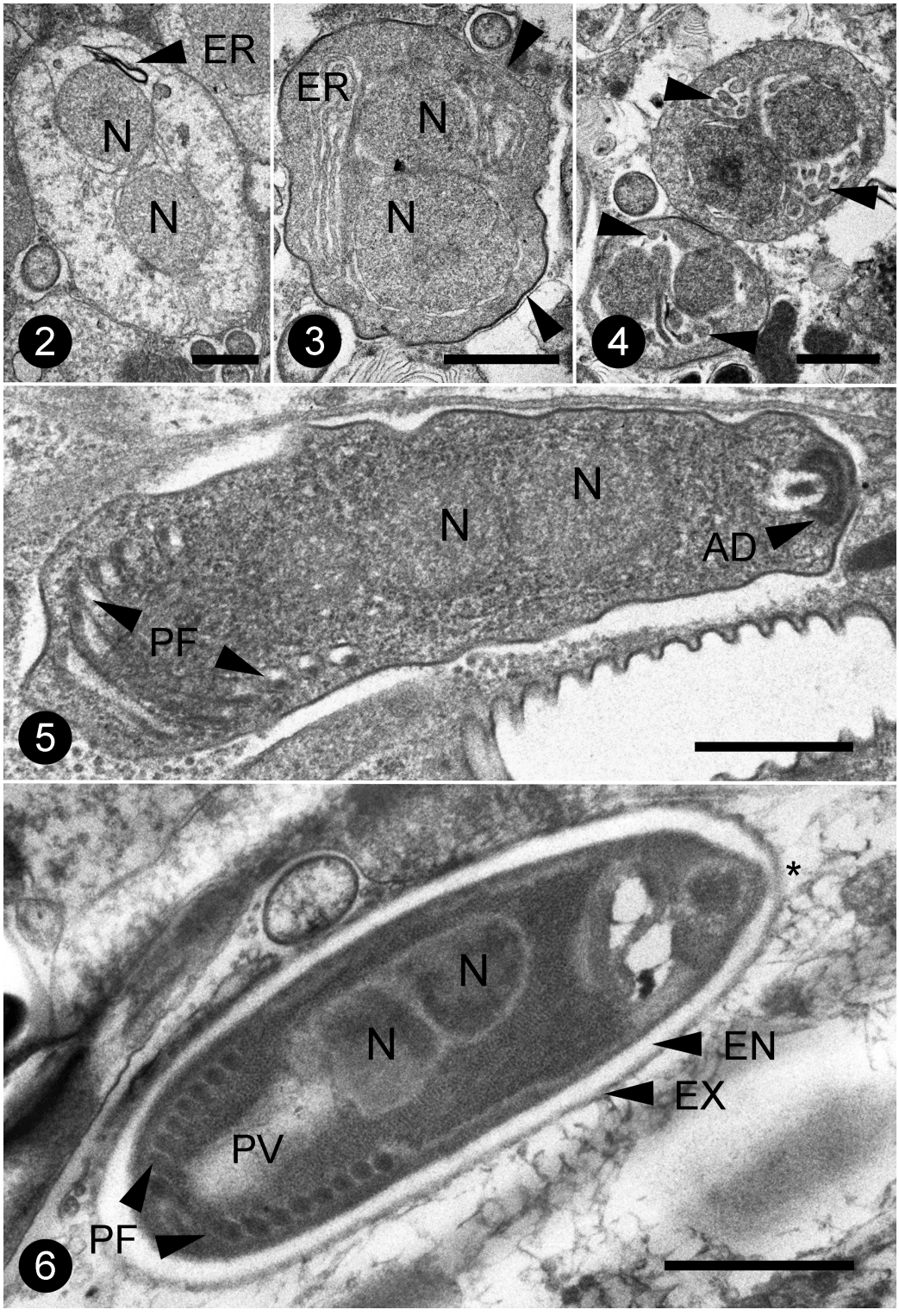
Fig. 2.3. Sporont with a developing thick outer membrane (arrows), well-developed endoplasmic reticulum (ER) and diplokaryon (N).

Fig. 2.4. Diplokaryotic sporonts showing tubular structures (arrows) within the cytoplasm.

Fig. 2.5. Sporoblast with a diplokaryon (N), showing development of the anchoring disk (AD) and polar filament (PF).

Fig. 2.6. Mature spore with a fully-developed cell wall, composed of an exospore (EX) and endospore (EN), a well-defined diplokaryotic nucleus (N), and an isofilar polar filament (PF) arranged in a single layer. A rather inconspicuous polar vacuole (PV) is evident within the posterior end of the spore. Thinning of the spore wall is visible at the spore apex (*).

Scale bars: 1 μm.


ER
N
N
2
ER
N
N
3
4
N
N
AD
PF
5
*
N
N
EN
EX
PV
PF
6

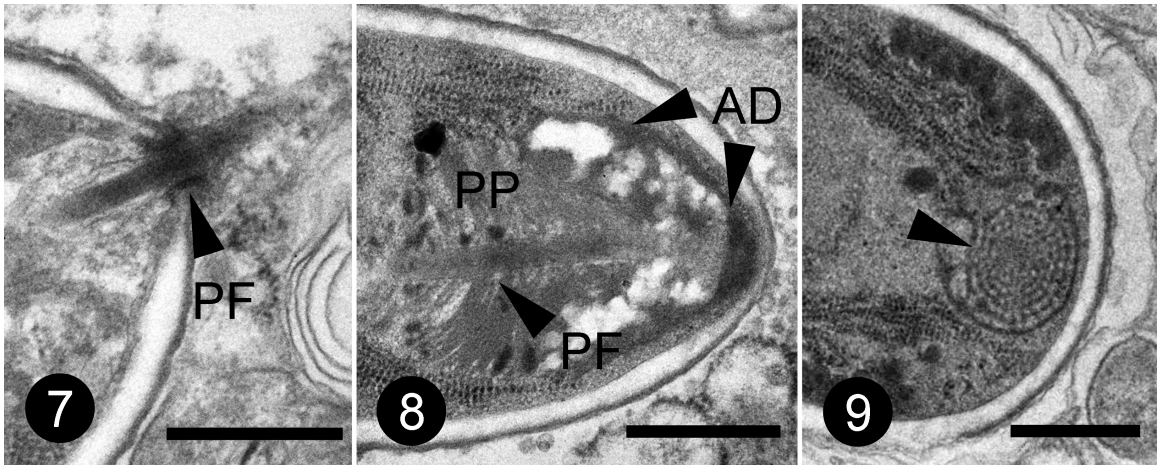
Figures 2.7 to 2.9. Ultrastructural detail of the anterior and posterior regions of mature spores of Nosema chrysoperlae sp. nov.

Fig. 2.7. Ejection of the polar filament (PF) through the apex of a mature spore.

Fig. 2.8. The anchoring disk (AD), lamellar polaroplast (PP) and polar filament (PF) located within the apical region of a mature spore.

Fig. 2.9. A cluster of small tubules (arrow) in the posterior region of a mature spore.

Scale bars: 0.5 μm.


PF
7
PP
AD
PF
8
9

Figs 2.10 to 2.13. Chrysoperla carnea tissues infected with Nosema chrysoperlae sp. nov.

Fig. 2.10. Microsporidian spores (arrows) within cells in the rectum.

Fig. 2.11. Microsporidian spores (arrows) in the peripheral region of the thoracic ganglion.

Fig. 2.12. Microsporidian spores within the flight muscles (arrows, left) and beneath the cuticle (arrows, right).

Fig. 2.13. Microsporidian spores (arrows) in the in the tissue surrounding the trachea.

Scale bars: 20 μm.

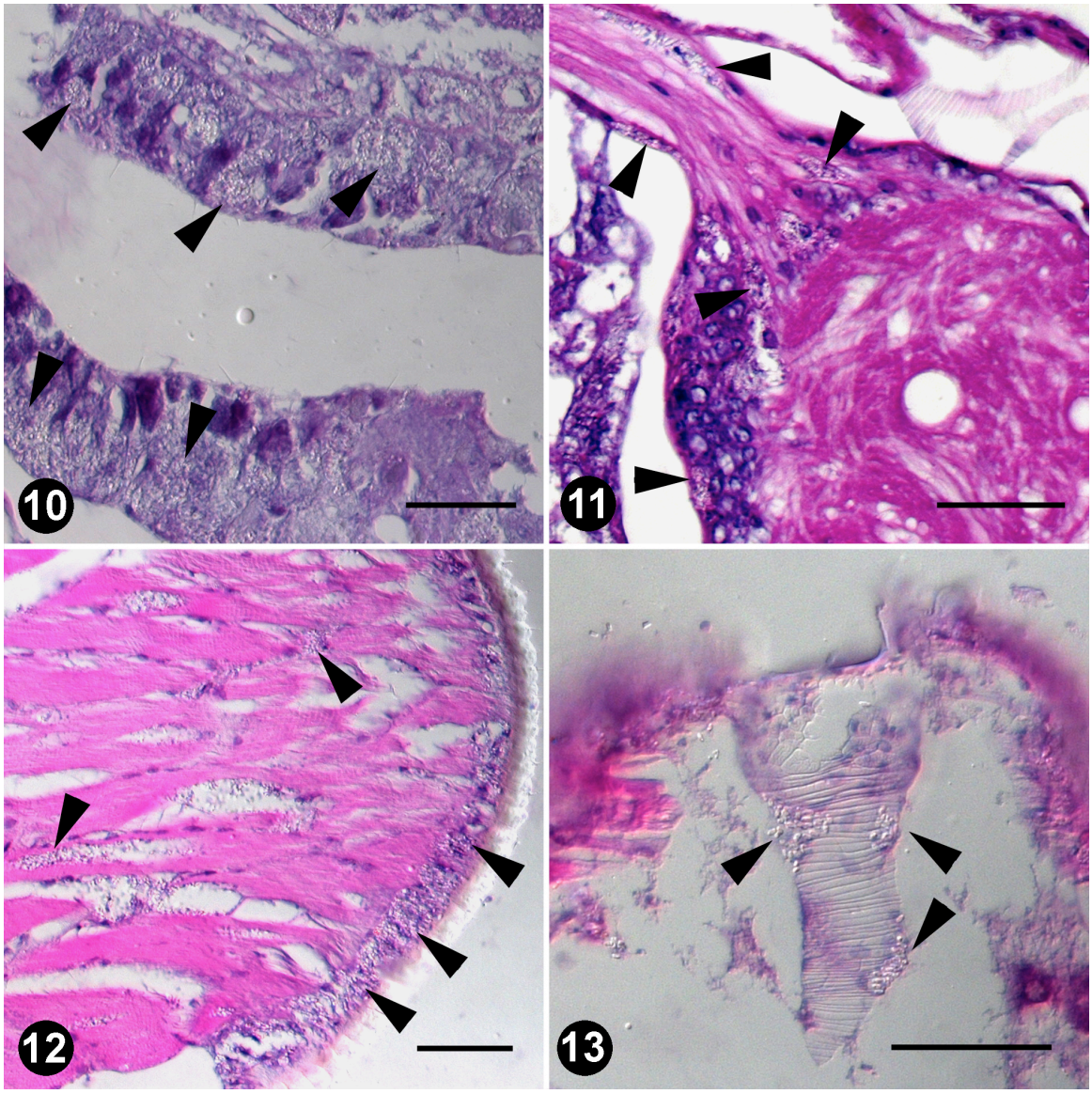
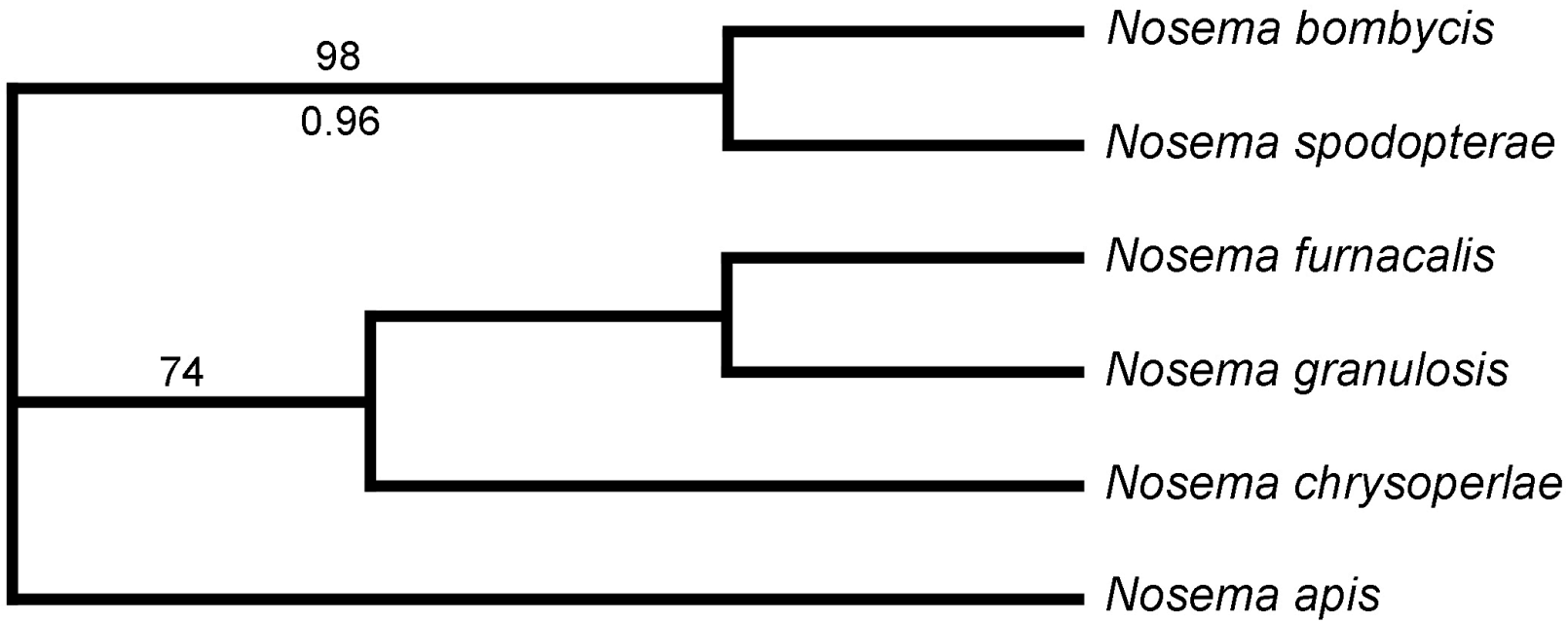

10
11
12
13

Fig. 2.14. Strict consensus tree derived from 16S ribosomal RNA gene sequence data conducted using a heuristic search with TBR branch swapping. Numbers above and below branches are bootstrap values and Bayesian posterior probability (PP) values, respectively. *Nosema apis* was used as an out group. Consistency index (CI) = 0.981, retention index (RI) = 0.743.


98
0.96
74
Nosema bombycis
Nosema spodopterae
Nosema furnacalis
Nosema granulosis
Nosema chrysoperlae
Nosema apis

CONCLUSION

Two new species of microsporidia found in beneficial insects were described; one from field collected *A. bipunctata* and the other from commercially available *C. carnea*. Formal descriptions of these microsporidian pathogens were based on pathogen ultrastructure, tissue pathology and molecular characterization. Transmission electron microscopy and light microscopy were used to examine the pathogen's ultrastructure and tissue pathology, respectively. Molecular characterization involved several techniques, including DNA extraction, genomic sequencing and the construction of phylogenetic trees.

The first pathogen described from the two-spotted lady beetle, *A. bipunctata*, was given the name *N. adaliae*. This microsporidium was 96% to 97% similar to several species within the genus *Nosema*, 96% similar to *O. occidentalis* and 95% and 96% similar to two species from the genus *Vairimorpha* (Table 1.1). This pathogen shared several characteristics with members of the genus *Nosema,* such as diplokaryotic spores and all stages in direct contact with the host cell cytoplasm. Mature spores measured 4.25 ± 0.09 x 1.82 ± 0.03 μm and had an isofilar polar filament with 10-18 coils arranged in single and double rows. Spores had a lamellar polaroplast, which was not typically visible, and a relatively small polar vacuole defined by electron-dense material along the internal perimeter. Numerous host tissues were infected including flight muscles and the fat body, which were heavily infected, as well as the ovaries, the testes, midgut epithelium, Malpighian tubules, ileum, colon, connective tissues and ventral nerve cord, which were not as heavily infected. The infected tissues in *A. bipunctata* are similar to

microsporidia described from other lady beetles, with the exception of *N. tracheophila* that primarily infects tracheal epithelium (see Table 1.3).

The second pathogen was described from the green lacewing, *C. carnea* and was given the name *N. chrysoperlae*. Molecular analysis of the microsporidian genome showed that the sequence was 99% similar to *Nosema granulosis*, *N. furnacalis*, *N. spodopterae* and *N. bombycis,* which confirms that this microsporidium belongs to the genus *Nosema.* The microsporidium described from *C. carnea* also shares similar characteristics to microsporidia belonging to the genus *Nosema*: all stages of the microsporidium were diplokaryotic and developed in direct contact with the host cell cytoplasm. Mature spores measured 3.49 ± 0.10 x 1.52 ± 0.05 μm and had an isofilar polar filament with 8-10 coils arranged in single and double rows. Spores contained a lamellar polaroplast and when observed, a relatively small and inconspicuous polar vacuole occupied the posterior region. Two distinct features were observed: (1) Tubular structures in both sporonts and in spores and (2) a cluster of small tubules in the posterior region of some mature spores. The majority of host tissues were infected. Microsporidian spores were observed in the proventriculus, diverticulum epithelial cells of the posterior midgut, Malpighian tubules, ileum, rectum, the fat body, peripheral region of the ganglia, within and between the flight muscles, and beneath the cuticle. Microsporidian spores were not observed within the developing eggs; this could explain why it was difficult to maintain microsporidia-infected *C. carnea* in the laboratory.

The hosts examined in this study, *A. bipunctata* and *C. carnea*, are both beneficial insects used in biological control programs. Although one pathogen is from field-

collected lady beetles and the other from commercially available lacewings, the presence of microsporidian pathogens in these natural enemies may have major implications with respect to the overall success of biological control programs. The microsporidium from local *A. bipunctata* could be vertically transmitted, *per os,* to imported *A. bipunctata* that are being used in biological control programs. Since microsporidia host specificity among lady beetles is not as narrow as previously thought, the pathogen could potentially be transmitted from *A. bipunctata* to other lady beetles that naturally share the same habitat or to lady beetles that are imported for biological control programs. This microsporidium has already been shown to significantly delay larval development in *A. bipunctata* (Steele and Bjørnson, 2012), however effects on other beetles are still unknown. In the case of *C. carnea,* some microsporidia-infected third-instar larvae died prematurely and larvae that survived either emerged as adults with malformed wings or were unable to eclose successfully. If these infected individuals were released in high numbers in a biological control program, one would expect fewer pests to be consumed and, since cannibalism is common among lacewings, infected individuals consumed by an uninfected could further spread the pathogen.

Although several microsporidia have been identified from beneficial insects (see Beerling and van der Geest, 1991; Geden et al. 1992; Bjørnson et al. 1996; Bjørnson, and Keddie, 2000; Becnel et al. 2002; Bjørnson et al. 2011), little is know about the potential impact of releasing these pathogens on the overall success of a biological control program. The microsporidium from *A. bipunctata* can significantly delay *A. bipunctata* larval development but does not appear to have any observable effects on adult fecundity

or longevity (Steele & Bjørnson, 2011). One possible explanation is that observations were made under laboratory conditions and individuals were provided with an ample supply of aphids and were kept in cups that limited their movements (Steele & Bjørnson, 2011). Future studies could determine if stressful conditions (limiting food availability and providing individuals with more space to move around) would result in observable effects on hosts. Further work could examine host range and what effects this microsporidium may have on other susceptible hosts. In the case of *C. carnea,* some infected larvae and adults showed distinct signs associated with infection but this was not studied in-depth since it was unfeasible to maintain infected *C. carnea* colonies in the laboratory. Future studies could focus on the prevalence of this pathogen in *C. carnea* from commercial insectaries, the correlation between symptoms and infection, effects on hosts, host range and pathogen transmission.

A growing concern regarding the overall success of biological control is the quality of natural enemies used. Until the 1980s, biological control agents were produced and distributed without any quality control procedures (van Lenteren, 2003). Quality control guidelines have since been established for many commercially available biological control agents, however the amount of screening and testing done varies depending on the size of the commercial insectary and the number of control agents produced (van Lenteren, 2003). Even when practiced, quality control outcomes are sometimes questionable since suppliers are often reluctant to release production information (van Lenteren, 2003). Quality control for *C. carnea* is also questionable since

one of the pathogens described in this study originated in insects purchased from a commercial insectary.

Quality control typically involves testing insects for any negative effects resulting from genetic variation, food supply (artificial diet, non target prey and target prey), abiotic conditions (temperature), behavioral variations and natural enemies (pathogens or parasites). The most common pathogens encountered in mass insect rearing are fungi followed by bacteria, viruses, protozoa and nematodes (van Lenteren, 2003). Some pathogens may affect the performance of natural enemies by lowering their efficacy, whereas others alter their ability to reproduce (van Lenteren, 2003). In order to obtain high efficacy in pest control, the release of pathogen-free natural enemies in biological control programs is of the utmost importance (see Bjørnson & Schütte, 2003). Elimination of pathogens from insect colonies is difficult, time-consuming and costly; therefore, it is important to screen individuals for pathogens on a routine basis (van Lenteren, 2003).

Although there have been some problems associated with biological control, such as cost, invasive species and the quality of natural enemies used, the benefits of this form of pest management often outweigh the risks. As more negative aspects of chemical pest control agents are becoming apparent, environmentally friendly alternatives such as natural enemies are increasing in popularity. Biological control agents are often target specific, can be self-propagating and, when compared to chemical control measures, are more environmentally friendly, less expensive and do not result in pesticide resistance. Natural enemies are an important part of integrated pest management (IPM) programs and

efforts should be made to ensure that natural enemies are free of pathogens to ensure successful pest control.

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Appendix A

Modified tissue preparation and embedment protocol for transmission electron microscopy (Becnel, 1997)

Fixation:

1) Dissect specimen in 2.5% glutaraldehyde (5 to 15 minutes)

2) Transfer dissected pieces into fresh 2.5% glutaraldehyde (2.5 hours to overnight)

3) Wash in 0.1M cacodylate buffer pH 7.2-7.3 (3 washes, 15 minutes each)

4) Post-fixation: 1% OsO_4 (pH 7.5) at room temperature (1 hour and 45 minutes to 2 hours, vials should be wrapped in foil)

5) Wash in double distilled water (3 washes, 15 minutes each)

Dehydration:

1) 10% EtOH (10 minutes)

2) 30% EtOH (10 minutes)

3) 50% EtOH (10 minutes)

4) 70% EtOH (10 minutes)

5) 80% EtOH (10 minutes)

6) 90% EtOH (10 minutes)

7) 95% EtOH (10 minutes)

8) 100% EtOH (2 washes, 15 minutes each)

9) 100% acetone (2 washes, 15 minutes each)

Embedding in Resin (1:1; Jembed 812:Spurr):

1) Resin + acetone (1:3, 4 hours to overnight)

2) Resin + acetone (1:1, 4 hours)

3) Resin + acetone (3:1, 4 hours)

4) 100% Resin (4 hours, overnight under vacuum at 15 PSI)

5) Pure resin (change vials) all day (6 hours)

6) Embed in capsules that have dried for at least 24hrs in 60°C oven

    a) add a small drop of fresh resin into tip of capsule

    b) place tissue into the drop; fill capsule with resin

7) Leave in oven overnight (uncovered)

8) Remove embedded blocks and allow to cool for 24 hours prior to sectioning

Post-staining:

1) Allow grids to completely dry (1 hour) and perform immediately prior to viewing

2) Float grids onto a drop of uranyl acetate (5 minutes)

3) Wash grids with de-ionized water (3 times, 5 minutes each)

4) Float grids on a drop of lead citrate, section side up (5 minutes)

5) Wash grids with de-ionized water (3 times, 5 minutes each)

6) Blot grids and wait for them to dry before viewing (1 hour)

Reference

Becnel J.J. 1997. Complementary techniques: Preparations of entomopathogens and diseased specimens for more detailed study using microscopy. In: Lacey L.A. (ed). Manual of techniques in insect pathology. Academic Press. pp. 337-353.

Appendix B

Modified protocol for embedment and sectioning of tissues in paraffin (Becnel, 1997)

Fixation:

1) Dissect specimen in Carnoy's fixative (60 ml absolute ethanol, 30 ml chloroform, 10 ml glacial acetic acid)
2) Soak speciments in 70% EtOH (overnight)

Dehydration:

1) 80% EtOH (2 hours)
2) 95% EtOH (2 hours)
3) 100% EtOH (2 washes, 1 hour each)
4) 100% EtOH + 100% butanol (1:1, overnight)
5) 100% butanol (above 25.5°C; 2 washes, 2 hours each)

Embedding:

1) 100% butanol + 100% Paraplast in 60°C oven (3:1, 20 minutes)
2) 100% butanol + 100% Paraplast in 60°C oven (1:1, overnight)
3) 100% Paraplast in 60°C oven (2 hours)
4) 100% Paraplast in 60°C oven under vacuum (15 PSI, 2 hours)
5) Embed in capsules made from paper folded into a rectangular container
    a) Add a shallow layer of fresh Paraplast into the bottom of capsule and let it harden slightly

b) Position tissues onto layer of Paraplast; fill capsule with Paraplast

c) Hold container on the surface of a cold water bath until a uniform film has formed on the paraffin

d) Submerge container in cold water and allow to harden

Sectioning:

1) Trim block to expose tissue

2) Make sections using rotary microtome; use paper and a fine brush to move sections

4) Mount sections to microscope slides

a) Coat slides with protein solution (1g gelatin, 2 g solid phenol, 15 ml glycerin, 100 ml distilled water)

b) Add a few drops of 1% formalin on slide

c) Float sections on formalin drops (carefully, use scalpel or brush)

d) Place slides on slide warmer (50°C) to flatten, straighten and fix sections

e) Leave slides on slide warmer (40°C) overnight to dry

Reference

Becnel J.J. 1997. Complementary techniques: Preparations of entomopathogens and diseased specimens for more detailed study using microscopy. In: Lacey L.A. (ed). Manual of techniques in insect pathology. Academic Press. pp. 337-353.

Appendix C

Rehydration and staining of slides using hematoxylin and eosin stains

Rehydration:

1) 100% xylene (2 washes, 2 hours each)

2) 100% xylene: 100% EtOH (1:1, 3 minutes)

3) 100% EtOH (3 minutes)

4) 95% EtOH (3 minutes)

5) 70% EtOH (3 minutes)

6) 50% EtOH (3 minutes)

7) Distilled water (3 minutes)

Staining:

1) Harris hematoxylin solution (filtered, 6 minutes)

2) Rinse in tap water (30 seconds)

3) Differentiating solution (0.25 HCl, 100 ml 70% EtOH; 3 dips)

4) Rinse in tap water (30 seconds)

5) Blue in Scott's tap water substitute solution (500 ml tap water, 5 g magnesium sulphate, 1 g sodium bicarbonate; 30 seconds)

6) 95% EtOH (2 washes, 1 minute each)

7) Alcoholic Eosin Y (30 seconds)

8) 100% EtOH (2 washes, 1 minute each)

9) 100% xylene (10 minutes)