Ultrastructure of an unidentified microsporidium from the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville

By

Jeffrey Le

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

September 15 2009, Halifax, Nova Scotia

Approved: Dr. Susan Bjornson
Supervisor
Associate Professor

Approved: Dr. Thomas Rand
Professor

Approved: Dr. Cristian Suteanu
Associate Professor

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Examining Committee:

Approved: Dr. Leellen Solter, External Examiner

Department of Entomology, University of Illinois

Approved: Dr. Susan Bjornson, Senior Supervisor

Department of Biology

Approved: Dr. Thomas Rand, Supervisor Committee Member

Department of Biology

Approved: Dr. Cristian Suteanu, Supervisor Committee Member

Department of Environmental studies

Approved: Dr. Ron Russell, Program Representative

Approved: Dr. Kevin Vessey, Dean of Graduate Studies

Date: September 15, 2009

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Abstract

An unidentified microsporidium was found in adult *Hippodamia convergens* Guérin-Méneville that were obtained from a commercial insectary for aphid biological control. Ultrastructure of the microsporidium was examined by transmission electron microscopy. All stages of the microsporidium were diplokaryotic and the pathogen developed in direct contact with host cell cytoplasm. Two morphologically distinct spores were observed. The first type had a thin exospore, thickened endospore, and typical internal spore structures. The second spore type was atypical in appearance, lacking any discernable internal structures. Typical and atypical spores measured 3.5 ± 0.2 x 2.06 ± 0.19 μm (n =10) and 3.38 ± 0.8 x 2.13 ± 0.16 μm (n =10), respectively. Based on spore dimensions and pathogen ultrastructure, the results of this study indicate that the microsporidium in question is likely *Nosema hippodamiae* Lipa & Steinhaus. Further evidence based on molecular technqiues is required for confirmation.

September 15, 2009

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Introduction

In 1919, H.S Smith introduced the term biological control to describe the use of predation, parasitism, herbivory, and other natural methods for the purpose of manipulating populations of organisms considered to be pests (Harris, 1997; Eilenberg et al., 2001; Dixon, 2000). Biological control incorporates many different fields of study such as microbiology, ecology and entomology, in order to maximize its effectiveness on target pests (Eilenberg et al., 2001). Currently, biological control is utilized in many agricultural settings including forests, glasshouse crops, and home gardens (Lacy et al., 2001).

There are four types of biological control. The first type is referred to as classical biological control, which entails the introduction of specialist natural enemies from the homeland of a pest of foreign origin. The objective is to establish populations of these natural enemies to attack the pest and to reduce its numbers. Augmentative biological control involves the mass rearing and release of large numbers of a particular biological control agent to supplement the small numbers that already exist in the local environment. Natural (passive) control is the use of an endemic species as the biological control agent (regardless of whether the target pest is endemic or introduced). Lastly, neoclassical biological control involves the importation of a biological control agent to suppress a native pest species (Vail et al., 2001).

Biological pest control is a popular choice for pest management because it does not rely on the application of harmful chemical agents that cause detrimental effects to both humans and pests (Simberloff, 1996). Many pesticides are broad spectrum with

respect to their mode of action and such chemicals may cause death or injury to non-target animal species. Chemical usage in agricultural or horticultural ecosystems may also induce harmful environmental impacts due to the trace chemical residues that are often left behind after application (Bunesu et al., 2002). Another disadvantage associated with chemical pesticide use is the risk of contamination to underground water reservoirs following application, resulting in the inadvertent poisoning of drinking water (Bucheit & Witzenbacher, 1996). Furthermore, pests are known to develop resistance to chemical insecticides when they are applied frequently.

Although many of the negative effects associated with pesticide use are not associated with biological pest control, the release of biological control agents for pest control can also cause concern. The safety of biological pest control to local ecosystem is still in question, as little data exists on the impacts that accompany the introduction of a new species into an ecosystem (Louda et al., 1997). Direct and indirect non-target impacts are unpredictable and thus a great cause for concern (Denslow et al., 2005). Interactions among native and introduced species may result in a variety of outcomes and these may be difficult to predict. Competition for resources, interruption of an established symbiosis with a another organism, or suppression of an organism which limits the distribution of its natural population, are but a few of the possible outcomes that may result from the introduction of a new species into a particular environment system (Hoogendoorn et al., 2002).

Use of lady beetles (Coccinellidae) for biological control

Coccinellids have been used for biological pest control for more than 100 years. Because of their predaceous nature, and the wide range of insects that they prey upon, many lady beetle species are used to suppress insect pests such as aphids, whiteflies, mealy bugs, scales and mites (Obrycki & Kring, 1998). Lady beetles are relative inexpensive and easy to apply.

The first recorded use of coccinellids as biological control agents (insect natural enemies) was the introduction of *Coccinella undecimpunctata* L. to New Zealand in 1815. The attempt to suppress native aphid populations was considered only moderately successful because *C. undecimpunctata* did not proliferate following release (Dixon, 2000). The first great success was documented in 1889 with the introduction of *Rodolia cardinalis* Mulsant to California in an attempt to suppress cottony cushion scale that was destroying local citrus crops (Dixon, 2000). The introduction of *R. cardinalis* was so successful and the pest was nearly eradicated (Dixon, 2000). This success sparked great interest in the use of lady beetles as biological control agents for the suppression of other insect pests; however, the desired result seen in California was not always reproducible due to the unpredictable interactions that occur between foreign and native organisms within a particular ecosystem.

Application of coccinellids as biological control agents is thought to be a simple process but there are several factors that affect the efficiency and overall success of lady beetles once they are released in the field. For example, exposure to pesticides and their residues may result in beetle mortality (Obrycki & Kring, 1998). Those that survive may

disperse to search of food and beetle longevity and fecundity may be greatly reduced as a result of chemical pesticide applications (Obrycki & Kring, 1998). Therefore, if both insecticides and lady beetles are simultaneously used for pest control, the timing and dosage of pesticide applications is key to lady beetle survival and efficacy.

Microsporidia and the convergent lady beetle, Hippodamia convergens

As the most prevalent lady beetle used for biological control in North America, the importance of the convergent lady beetle, *Hippodamia convergens* Guérin-Méneville cannot be overstated. *H. convergens* has been used for nearly a century for aphid control in agriculture (Carnes, 1912). In its role as a research model, *H. convergens* is being used to elicit the identity of a previously undescribed microsporidium, providing clarity to the unstable and ever-changing taxonomic classification and organization of these protistan pathogens.

Microsporidia are obligate-intracellular parasites that are known to produce chronic, debilitating disease within their respective hosts. Originally identified as protozoa, recent molecular evidence gathered from 8 genes representing 4 fungal phyla (see Gill & Fast, 2006) suggests that microsporidia are closely related to fungi (sister to a shared ascomycete and basidiomycete clade) (Gill & Fast, 2006). However, molecular sequencing of RPB1 (DNA –dependant RNA polymerase II largest subunit) and EF-1a (an elongation factor) suggest that a relationship between microsporidia and fungi is lacking (Tanabe *et al.*, 2002). It is clear that a relationship exists between microsporidia and fungi; however, the exact nature of the relationship is yet to be resolved.

Microsporidia are prevalent pathogens among animals, but are more commonly found in arthropods and fish (Keeling & Fast, 2002). Discovered in the nineteenth century during an outbreak of pebrine disease that was ravaging the silk industry in southern Europe, K. Nageli identified the agent inducing the disease as *Nosema bombycis* and classified it with the schizomycete fungi (Keeling & Fast, 2002). Further study of *Nosema bombycis* by Balbiani prompted the creation of the new phylum Microsporidia in 1882, which is still in use today (Keeling & Fast, 2002).

Until 1985, microsporidia were not considered to be important with respect to human medicine. However, with the isolation of *Enterocytozoon bienusi* in AIDs-infected humans, and the AIDS pandemic of the 1980s whereby large populations of people became infected with the HIV virus, microsporidia gained much more attention (Weber et al., 1994). Further research revealed that microsporidioses was not a disease of humans infected with AIDs as it was once suspected, but of those who were immunocompromised (for example, organ transplant recipients, cancer patients receiving chemotherapy) (Dider et al.2005).

The origin of microsporidia was once a subject of much debate. Microsporidia lack key cellular structures, including mitochondria, centrioles and peroxisomes, which are ubiquitous to eukaryotic cells (Keeling & Mcfadden, 1998). Therefore, microsporidia were initially classified as Archezoa. However, with the advent of molecular techniques as a tool to characterize microsporidia (by sequencing ribosomal RNA), microsporidia are now thought to be closely related to fungi (Keeling & Mcfadden, 1998). Other evidence in support of this fungal relationship is the discovery of relics of the heat shock

protein HSP 70. This protein is found in association with eukaryotic mitochondria and its discovery in microsporidia suggests that these pathogens lost their mitochondria early on with respect to the evolution of eukaryotic organisms (Germot et .al, 1997; Keeling & Mcfadden, 1998; Keeling & Naomi, 2002).

In general, microsporidioses induce a large array of symptoms in invertebrates, including morphological abnormalities, changes in color, and lethargic or abnormal behaviour (Tanada & Kaya, 1993; Bjørnson & Keddie, 1999; Boohene, et al. 2003; Olsen & Hoy, 2002). Other symptoms of microsporidia-infected *H. convergens* are subtle and include prolonged larval development, reduced fecundity and reduced female longevity (Saito & Bjornson, 2006; Joudrey & Bjornson, 2006).

Three species of microsporidia have been reported from coccinellids in past studies. The microsporidium *Nosema hippodamiae* Lipa and Steinhaus was reported from *H. convergens* in 1959 (Lipa & Steinhaus, 1959). In later studies, *Nosema tracheophila* Cali and Briggs was reported from *Coccinella septempunctata* L. (Cali & Briggs, 1967) and *Nosema coccinellae* Lipa was reported from *C. septempunctata* and other field-collected coccinellids (Lipa, 1968). All three described species of microsporidia (*N. hippodamiae*, *N. tracheophila* and *N. coccinellae*) share similar morphological characteristics in regards to spore shape and size. However, some variability is observed in regards to tissue specificity. For example, *N. hippodamiae* infects the midgut epithelium and fat body of *H. convergens* whereas *N. tracheophila* infects the tracheal epithelium, blood cells, and connective tissue. *N. coccinellae* infects nerves, midgut,

gonads, muscle, and the Malpighian tubules (Cali & Briggs, 1967; Lipa, 1968; Lipa & Steinhaus, 1959).

In 2004, an unidentified microsporidium was found in adult *H. convergens* that were obtained from a commercial insectary for aphid biological control. Within a laboratory setting, this microsporidium was transmitted horizontally among several non-target coccinellids, including the two-spotted lady beetle (*Adalia bipunctata* L.), the seven-spotted lady beetle (*C. septempunctata*), the two-banded lady beetle (*C. trifasciata perplexa* Mulsant), and the multicoloured Asian lady beetle (*Harmonia axyridis* Pallas) (Saito & Bjørnson, 2006, 2008). Although *N. hippodamiae* was reported from *H. convergens* by Lipa in 1959, it is unclear if the unidentified microsporidium in *H. convergens* is *N. hippodamiae.*

Successful horizontal transmission of the unidentified microsporidium from *H. convergens* to other non-target coccinellids (Saito & Bjørnson, 2006, 2008) suggests that this microsporidium may have a relatively broad host range. The unidentified microsporidium in *H. convergens* has spores that are similar in size to those of *N. hippodamiae*, *N. tracheophila* and *N. coccinella.* The considerable overlap of tissues infected with these microsporidia, and their similar spore dimensions, raises questions regarding the true identity of the microsporidia from coccinellids. It is possible that what is currently thought as three distinct species of microsporidia (*N. hippodamiae*, *N. tracheophila* and *N. coccinellae*), are actuality a single species that has a relatively broad host range in coccinellid beetles.

Symptoms of microsporidioses are difficult to observe and identify in living hosts (Tanada & Kaya, 1993). Thus, diagnosis in most invertebrates is performed post-mortem. Microsporidian spores are observed when smear preparations of infected specimens are stained with Giemsa (a dichromatic spore stain used to indicate the presence of microsporidian spores) and examined by light microscopy. However, pathogen ultrastructure and development are best observed by examining ultra-thin tissue samples using transmission electron microscopy. Although a description of microsporidian development and mature spore characteristics are used for species identification (Becnel & Geden, 1994), molecular characterization is required for a formal description of the pathogen (Becnel et al. 2005).

The life cycle of microsporidia involves two phases: the proliferation of vegetative stages within host cells (merogony) and the dissemination of infective spores that are able to withstand environmental conditions outside the host (sporogony). Microsporidian spores are usually able to infect other susceptible hosts when ingested. The physical characteristics of microsporidian spores are variable and both internal and external features are used for pathogen identification (Tanada & Kaya, 1993). Spores are described in terms of their shape, dimensions, and internal spore characteristics. The mature spore is comprised of three basic components: the sporoplasm, the polar filament (a tube-like extrusion apparatus) and the spore wall (Tanada & Kaya, 1993). Spore discharge occurs as a response to the osmolarity of the surrounding environment within the host gut lumen (Weidner, 1976). Once the coiled polar filament is released, it

penetrates the membrane of an adjacent host cell and acts as a mechanism for injecting the infective sporoplasm into it.

The sporoplasm undergoes extensive multiplication by merogony (binary fission) or schizogony (multiple fission) and sporogony (spore production) occurs once multiplication of the sporoplasm is complete. This process ensures that the vegetative cells of the microsporidium replicate in large numbers and develop into viable infective spores. In some types of microsporidia, such as *Nosema*, when spores reach a critical number, the host cell membrane is disrupted and the spores are released (Tanada & Kaya, 1993). However, depending on the species of microsporidium, spores may be released through differing mechanisms. Some spores are capable of autoinfection (infection of different cells in the same host) whereas others are transmitted to new hosts (horizontal transmission). Microsporidia are also transmitted vertically from parent to offspring (Tanada & Kaya, 1993).

Different species of microsporidium posses a particular type of life cycle that is characteristic of their species. For example, microsporidia of the genus *Nosema* undergo development in direct contact with the host cell cytoplasm and its proliferative phases (merogony and sporogony) divide by binary fission (Sprague et al, 1992). In contrast, microsporidia of the genus *Vairimorpha* undergo development similar to *Nosema* but proliferative stages do not develop indirect contact with host cell cytoplasm. Rather, a sporophorus vesicle surrounds the developmental stages and mature spores. Instead of having a single sporogonial stage, *Vairimorpha* undergoes two separate avenues of

sporogonial development, resulting in two ultrastructurally distinct spores (heterosporous) (Vavra et al, 2006; Becnel, 1994).

The development of two types of spores may be used by the pathogen for specific modes of transmission. For example, the microsporidium *Thelohania legeri*, from the mosquito host *Anopheles maculipennis* is heterosporous, with each spore responsible for its own task (one type of spore for autoinfection, the second type for horizontal transmission) (Becnel, 1994).

In some cases, microsporidia may induce physiological changes to the host cell, including the re-arrangement of organelles such as mitochondria and endoplasmic reticulum to surround life cycle stages, and the enlargement of the host cell (xenoma) due to the presence of numerous spores (Canning & Curry, 2005; Keeling and Fast, 2002).

Although an estimated 1200 species of microsporidia have been described (Sprague et .al, 1992, Keeling & Fast, 2002), Phylum Microsporidia is constantly undergoing taxonomically reclassification. Past descriptions based solely on information from light microscopic observations, and the discovery of undescribed microsporidia, complicate the classification of these important pathogens. The objective of this study is to describe the ultrastructure of an unidentified microsporidium from the convergent lady beetle, *Hippodamia convergens*. Molecular characterization, also required for the complete classification of this pathogen, will be the focus of another study. An ultrastructural description of the pathogen will help provide a foundation for future studies of microsporidia from *H. convergens* and other predaceous coccinellids that are used for biological control. An increased understanding of microsporidian pathogens and

microsporidiosis will help ensure that beneficial arthropods remain a viable alternative to chemical pest control.

Materials and Methods

Insect Rearing

Nasturtium (*Tropaeolum nanum* Jewel Mixed, Stokes Seeds, ON) were seeded and maintained under controlled conditions (Sanyo Environmental Chambers) (25°C, 16L:8D). These plants were used to rear colonies of green peach aphids (*Myzus persicae* Sulzer), which were used as food for *H. convergens*.

Uninfected and microsporidia-infected *H. convergens* larvae were reared in polyethylene cups and fed an *ad libitum* diet of aphids, distilled water, and Lacewing and Ladybug Food (Natural Planet Lacewing Food, Planet Natural, MT) that was mixed with honey and water (ratio: 20 mL, 20 mL, 1 mL). Beetles were reared and maintained under controlled conditions (25°C, 16L:8D).

Sample preparation

H. convergens was confirmed as uninfected or microsporidian-infected through the examination of Giemsa stained smears of eggs obtained from parent females (see Saito & Bjornson, 2008). Twelve specimens (6 uninfected and 6 infected) were processed simultaneously. This process was repeated six times (total specimens: 36 uninfected and 36 infected) on different dates. To maximize penetration of the fixative, the elytra, legs, and thorax from each specimen were removed. The abdomen was then divided into 6 sections (one longitudinal cut and two lateral cuts across the abdomen) while the

specimen was submerged in 2.5% gluteraldehyde. Tissues were embedded in Epon 812/Spurr resin. Sample fixation and embedment were performed according to a method widely used for the examination of microsporidia in arthropod hosts (Becnel, 1997; see Appendix). A small adjustment to the protocol was made by placing the specimens under vacuum (15 PSI) while in 100% resin.

Tissue blocks were chosen at random for examination. A Leica UCT ultramicrotome was used to cut semi-thin and ultra-thin sections. Semi-thin sections were placed onto glass slides, heat dried then stained with toluene blue, then washed with methanol. These were examined for microsporidian spores by light microscopy. Ultra-thin sections were placed onto formvar-coated grids and subsequently stained with uranyl acetate and lead citrate. Procedures used for preparating tissues and TEM are provided in the Appendix.

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

Results

All stages of the microsporidium were observed in direct contact with the host cell cytoplasm. Life cycle stages were proximal to mature spores and both were observed throughout the tissue sections that were examined. All developmental stages were diplokaryotic; however, diplokarya were not observed in all cells. Even when diplokarya

were observed, the nuclear membrane that defined the twin nuclei of the diplokarya was not always prominent.

The round to slightly ovoid-shaped sporoplasm (Fig. 1) was surrounded by a thin plasma membrane. Internally, the cytoplasm possessed many free ribosomes and evidence of a weakly developing endoplasmic reticulum. The diplokaryon occupied approximately two-thirds of the cell and condensed chromatin was observed occasionally within the diplokaryon.

Meronts (Fig. 2) were surrounded by a plasma membrane. Free-floating ribosomes were observed within the merogonial cytoplasm. The endoplasmic reticulum, observed around the perimeter of the diplokaryon, was slightly more prominent in the merogonial cytoplasm than it was in the sporoplasm. Meronts proliferated by means of binary fission (Fig.3)

Sporonts were surrounded by an electron dense membrane (Fig. 4) and the sporogonial cytoplasm contained a well-developed and prominent endoplasmic reticulum. Sporonts contained fewer free ribosomes within the cell cytoplasm than did meronts, and as a result, the sporogonial cytoplasm appeared less dense than did the merogonial cytoplasm.

Two distinct types of sporoblasts were observed. The first type of sporoblast (Fig. 5) was surrounded by an irregular-shaped plasma membrane that often appeared highly convoluted. The cytoplasm of these cells was electron dense and although cell nuclei were observed frequently within these sporoblasts, the nuclei were not always observed as distinct diplokarya. The second type of sporoblast (Figs. 6 and 7) was characterized by

a relatively complete spore wall that surrounded a round to somewhat elongated cell. The polar filament, polaroplast, and anchoring disk were readily observed within the cell cytoplasm where these structures appeared to undergo various stages of development and organization. The exospore appeared to develop at the same time as the polar filament, whereas the endospore, along with other internal spore structures such as the polaroplast and polar vacuole, developed afterward. Cell nuclei were not observed within these sporoblasts.

Two morphologically distinct spores were observed. The first type had a thin exospore, thickened endospore (exospore + endospore was 0.32 μm; Table 1), and typical internal spore structures (Figs. 8-10). A prominent indentation was evident at the apical end of the spore wall (Fig. 9) where polar filament discharge occurs. An isofilar polar filament, arranged in a single or double layer, possessed 10-14 coils (n = 10) that were arranged perpendicular to the longitudinal axis of the spore. The diplokaryon occupied approximately two-thirds of the spore and was surrounded by rough endoplasmic reticulum. A lamellar polaroplast occupied about one-third of the anterior region of the spore (Fig. 8) and a polar vacuole occupied approximately one-third of the posterior region of the spore. However, the polar vacuole was not observed within all spores that were examined. The second spore type (Figs 11-14) was atypical in appearance. These spores had a fully developed exospore but lacked any discernable internal spore structures. Instead, they were filled with lamellar or vesicular structures. Atypical spores were observed as often as were typical spores (proximal to each other), and both were observed throughout all tissues examined. Wall thickness of atypical spores was 0.27μm

(Table 1). Typical and atypical spores measured 3.58 ± 0.2 x 2.06 ± 0.2 μm (n =10) and 3.38 ± 0.8 x 2.13 ± 0.2 μm (n =10), respectively. The area (representative of the maximal area) of both typical and atypical spores was 6.42 ± 2.7 and 7.20 ± 1.2 μm^2, respectively (Table 1).

Discussion

The microsporidian life cycle that was observed during this study fulfils the criteria set forth by Sprague *et al.* (1992) for the classification of *Nosema bombycis* Naegeli, the type species for the genus *Nosema.* The microsporidium characterized herein from the host *Hippodamia convergens* is believed to be *Nosema hippodamiae* Lipa and Steinhaus (Lipa & Steinhaus, 1959). Although the description of *N. hippodamiae* is based solely on observations (life cycle, tissue pathology and spore dimensions) made by light microscopy, the description of *N. hippodamiae* bears considerable resemblance to what has been observed for the microsporidian pathogen that was the focus of this study.

Meronts of the microsporidium in this study proliferated by means of binary fission (merogony). In the case of *N. hippodamiae*, their pathogen is reported to undergo schizogony (Lipa & Steinhaus, 1959), a process that is defined broadly as proliferation by means of binary or multiple fission (Sprague *et al.* 1992). Schizogony, as described by Lipa and Steinhaus for *N. hippodamiae*, involves the division of schizonts into two daughter cells (binary fission). Therefore, their use of the term schizogony by Lipa and Steinhaus is synonymous with what is referred to herein as merogony.

Furthermore, spore shape and size of the unidentified microsporidium are consistent with what is reported for *N. hippodamiae*. With respect to tissue pathology, *N. hippodamiae* infects the midgut, fatbody and, depending on severity of infection, other surrounding tissues of *H. convergens*. In comparison, the unidentified microsporidium in *H. convergens* infects several tissues (Saito, 2008) and exhibits considerable tissue overlap with what is reported for *N. hippodamiae*. One notable difference is that *N. hippodamiae* is reported to infect the midgut, whereas the unidentified microsporidium does not infect the midgut but only the muscle tissue surrounding it (Lipa & Steinhaus, 1959; Saito, 2008). This difference may be attributed to the study techniques that were employed (smear preparations and dissections by Lipa & Steinhaus; tissue sections by Saito) and may therefore prove to be academic.

Microsporidian life cycle

The sporoplasm (Fig. 1) was surrounded by a thin plasma membrane and the presence of many free-floating ribosomes within the cytoplasm gave the cell an overall electron-dense appearance. In the case of *Glugea hertwigi* and *Spraguea lophii*, Weidner *et al.* (1984) demonstrated that the microsporidian sporoplasm obtains its plasma membrane from the polaroplast during spore discharge. It is assumed that the sporoplasm of *N. hippodamiae* obtains its plasma membrane through a similar process. Condensed chromatin, observed occasionally within the diplokaryon, is evidence of sporoplasm division.

Merogony is a proliferative phase responsible for a numerical increase of the pathogen within the host cell (Wang & Chen, 2007). Meronts (Fig. 2) were observed

through the host tissue and replicated by means of binary fission (Fig. 3). Meronts displayed characteristics that are typical of microsporidia undergoing merogony: an increase in electron density of the plasma membrane relative to that of the injected sporoplasm, the presence of free-floating ribosomes, an increase in the development of the endoplasmic reticulum, a defined nucleus, and an absence of Golgi bodies (Wang & Chen, 2007, Tanada & Kaya, 1993). Condensed chromatin was often observed within one or both diplokarya and its presence may indicate that either the host cell was about to divide or it had just undergone cell division.

The observation of an electron dense plasma membrane during the sporogonial stage (Fig. 4) is consistent with the typical morphological changes that occur during sporogony (Sprague *et al.* 1992). The sporogonial cytoplasm appears relatively less electron-dense than does the merogonial cytoplasm. This is due to the presence of a well-developed rough endoplasmic reticulum (RER). The RER provides a scaffold by which free floating ribosomes bind, causing an increase in the electron-lucence of the cytoplasm in the post- merogonial developmental stages (Vavra et al. 2006). An increase in sporogonial RER is an expected cellular event, considering the vast structural development that occurs during the transition from sporont to sporoblast.

Within the microsporidian life cycle, sporoblasts are readily distinguishable from any other developmental stage. Key sporoblast characteristics include the presence of a developing spore wall (comprised of the exospore and endospore), along with the development of internal structures, such as the polar filament and polaroplast (Tanada & Kaya, 1993). Sporoblasts with two distinct morphologies were observed (Figs.5-7).

The development of internal structures within the sporoblast (Figs 6, 7) seems to follow a distinct sequence. A well-developed exospore appears to develop at the same time as the polar filament. Once the exospore and polar filament are near completion, the endospore begins to develop along with the polaroplast and polar vacuole.

The mature spore wall is composed of a relatively thick endospore and a thinner exospore. An indentation at the apical end (Fig. 9) permits polar filament extrusion during spore discharge. Typically, the endospore is composed of a protein/chitin complex. This layer provides the spore with the structural rigidity required to withstand the change in hydrostatic pressure that accompanies sporoplasm extrusion (Tanada & Kaya, 1992). Mature spores (and other life cycle stages) (Figs. 8–10) were observed in large numbers throughout the host tissues and were in direct contact with host cell cytoplasm. This observation was supported by Saito (2008), who reported the occurrence of microsporidian spores in several host tissues, including the fatbody, muscles, Malpighian tubules, pyloric valve epithelium, muscle surrounding the midgut, nerves and gonads.

The presence of mature spores with unidentifiable internal structure is an interesting and puzzling observation. The location of microsporidian development within host tissue may explain why some spores develop atypically. In the case of the microsporidium *Vairimorpha disparis*, mature spores fail to mature in specific tissues of the gypsy moth, *Lymantria dispar* (Vavra et al. 2006). Although this may also be the case for the microsporidium in *H. convergens,* it seems an unlikely scenario because atypical spores were found interspersed with typical spores throughout all host tissues that were

examined. Alternatively, the presence of atypical spores may provide circumstantial evidence that *H. convergens* is not the natural host for this microsporidium. Under laboratory conditions, the microsporidium in *H. convergens* is transmitted to several coccinellid species, including *Adalia bipunctata*, *Coccinella septempunctata, C. trifasciata perplexa,* and *Harmonia axyridis* (Saito & Bjørnson, 2008). Although the microsporidium was originally found in *H. convergens* obtained from a commercial insectary, this pathogen may be native to another coccinellid host with tissues that are more conducive to its development. If this is the case, the tissues of *H. convergens* may not provide an environment that is adequate for supporting the development of all spores to maturity.

Alternatively, the formation of atypical spores by this microsporidium may be part of the natural life cycle of the pathogen. Atypical spores may eventually develop internal structures that are characteristic of typical microsporidian spores, taking more time to develop than typical spores. The production of atypical spores may be an adaptation of the microsporidium to better withstand *H. convergens* diapause (overwintering).

Spore size, shape (3.5 ± 0.2 x 2.06 ± 0.19 μm (n =10); ovoid) and tissue pathology of the microsporidium observed during this study was consistent with that reported for *N. hippodamiae* and for other microsporidia from coccinellids hosts (Table 1). Mature *N. hippodamiae* spores are ovoid and range 3.3-2.5 x 2.2-2.7 μm (Lipa & Steinhaus, 1959). Sluss (1966) later reported a microsporidium in from *H. convergens* with similar spore shape and dimensions that the author referred to as *N. hippodamiae.* In

contrast, mature spores of the microsporidium described herein are generally ovoid and measure 2.4-3.6 μm x 2.0-2.4 μm when fixed with methanol and stained with Giesma (Joudrey & Bjørnson, 2007). This microsporidium infects several tissues (Saito, 2008) that are also infected by *N. hippodamiae* (Sluss, 1966; Lipa & Steinhaus, 1959). Although there is slight variation in spore dimensions among the microsporidia that infect coccinellids, this could be attributed to differences in fixation methods and study techniques.

Observations with respect to host range, spore size and infected tissue overlap among *N. hippodamiae*, *N. tracheophila* and *N. coccinellae* have raised the possibly that what is currently thought of as three distinct species of microsporidia may actually be a single species. If this is the case, this microsporidium would possess a relatively broad host range and be capable of infecting several coccinellid hosts. Molecular characterization of the undescribed microsporidium must be completed and results compared to those from *N. hippodamiae*, *N. tracheophila* and *N. coccinellae* in order to confirm if this is the case. This may not be possible; however, because type specimens of these microsporidia may not exist. Confirmation of pathogen identity will provide clarification regarding the unstable and ever-changing taxonomic classification and organization of these fungal pathogens.

Pathogen Summary

Type host:

Hippodamia convergens Guérin-Méneville (Coleoptera: Coccinellidae)

Transmission:

Vertical transmission (transovarial) and horizontal transmission (healthy adults are infected with spores via ingestion). Reinfection of host tissues occurs following the germination of spores within host cells (autoinfection).

Site of infection:

Numerous tissues, including the fatbody, pyloric valve epithelium, muscle surrounding the midgut, nerves, gonads, muscle, Malpighian tubules (Saito, 2008).

Interface:

All microsporidian stages develop in direct contact with the host cell cytoplasm.

Other pathogen-host cell relations:

Pathogen is distributed randomly within the host cell cytoplasm.

Sporoplasm:

Sporoplasm possessed a thin plasma membrane and diplokaryotic nucleus that occupied two thirds of the cell. Free floating ribosomes were present, but no other internal structure was observed.

Meront:

Diplokaryotic nucleus is present. Internal structures such as endoplasmic reticulum continue development. Multiplication occurs through binary fission.

Sporont:

A thick, plasmodial membrane surrounds the diplokaryotic nucleus. The sporogonial cytoplasm appears less dense than does the merogonial cytoplasm. The endoplasmic reticulum is prominent and well defined.

Sporoblast:

Deposition of the exospore occurs at the same time that the endospore begins development. Development of typical internal structures such as the polar filament, lamellar polaroplast, and polar vacuole is observed. Sporoblasts appear dense and plasmodial in shape or round to somewhat elongated. A polaroplast, polar filament, and anchoring disk are readily observed.

Mature spore:

Spores were diplokaryotic and ovoid. Some spores possessed typical microsporidian internal structures. These measured 3.5 ± 0.2 x 2.06 ± 0.19 μm (n =10). An isofilar polar filament was arranged in 10-14 coils (n = 10) that was usually observed in a single layered arrangement. The polaroplast was lamellar in form. Atypical spores were also observed. These possessed unidentified internal structures and measured 3.38 ± 0.8 x 2.13 ± 0.16 μm (n =10).

Type locality:

The microsporidium was isolated from *Hippodamia convergens* that were purchased from a commercial insectary. The source of origin is likely the Sierra Nevada Mountains of Southern California where *H. convergens* is collected *en masse* for distribution as biological control agents.

Table 1

Spore measurements of an unidentified microsporidium within the convergent lady beetle, *Hippodamia convergens*

	Typical Spores	Atypical Spores
Spore wall thickness (μm ± SD)	0.32 ± 0.04	0.27 ± 0.08
Spore length (μm ± SD)	3.58 ± 0.2 x 2.06 ± 0.2 μm	3.38 ± 0.8 x 2.13 ± 0.2 μm
Spore area (μm^2 ± SD)	6.42 ± 2.7	7.20 ± 1.2

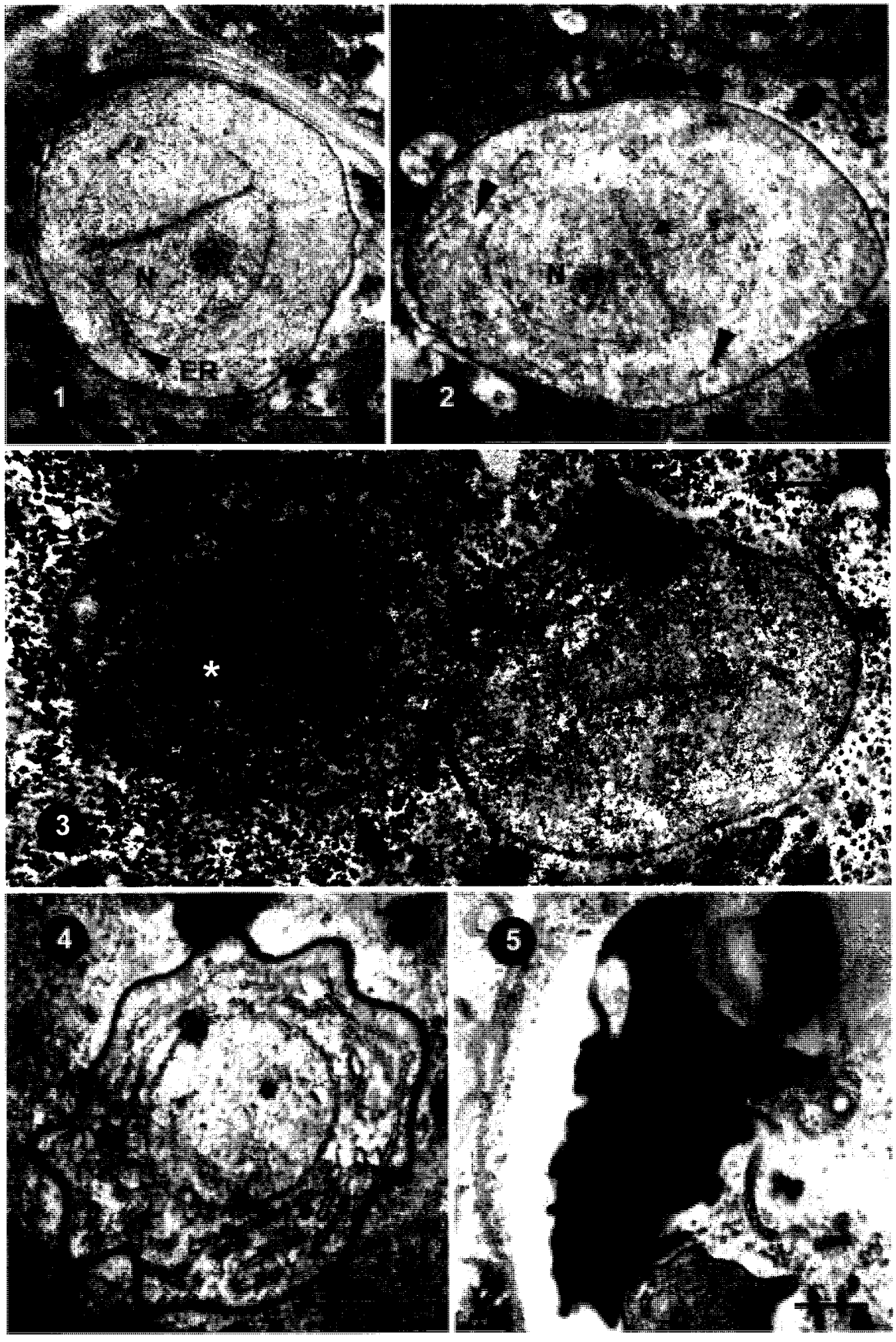
n = 10 for both typical and atypical spores. Typical spores are those with visible internal structures that are characteristic for microsporidia. Atypical spores lacked any discernable internal structures.

Table 2

A comparison of microsporidia from coccinellid hosts

Microsporidian Species	Spore Shape	Spore Size (length x width; μm)	Tissues Infected	Host Species	Authors
Nosema. hippodamiae	Oval	3.5-5.4 x 2.2-2.7[a] 2.9-3.8 x 1.4-1.9[1,b]	Midgut, fat body *Fat body, muscle tissue, Malpighian tubules, testes	*Hippodamia convergens*	Lipa & Steinhaus Sluss[1]
N. tracheophila	Oval	4-5.3 x 2.2-3.1[a]	Tracheal epithelium, blood cells, connective tissue	*Coccinella septempunctata*	Cali & Briggs
N. coccinellae	Oval	4.4-6.7 x 2.3-3.4[a]	Nerves, midgut, gonads, muscle, Malpighian tubules	*C. septempunctata* *Hippodamia convergens* *H. tredecimpunctata* *Myrrha octodecimguttata*	Lipa
Unidentified microsporidia	Oval	2.4-3.6 x 2.0-2.4[a] 3.5 x 2.06[2,c] 3.38 x 2.13 (atypical spores)[2,c]	Fatbody, muscles, Malpighian tubules, muscle surrounding the midgut, pyloric valve epithelium, connective tissue, nerves and gonads	*Adalia bipunctata,* *C. septempunctata,* *C. trifasciata perplexa* *Harmonia axyridis* *Hippodamia convergens*	Saito Le (current study)[2]

[a] tissue specimen fixed in methanol and stained with Giemsa; [b] whole beetle sections fixed in Gilsons, Bouins, Weaver-Thomas and Carnoy's; c resin embedded tissues fixed in paraformaldehyde and post-fixed in osmium tetroxide (current study)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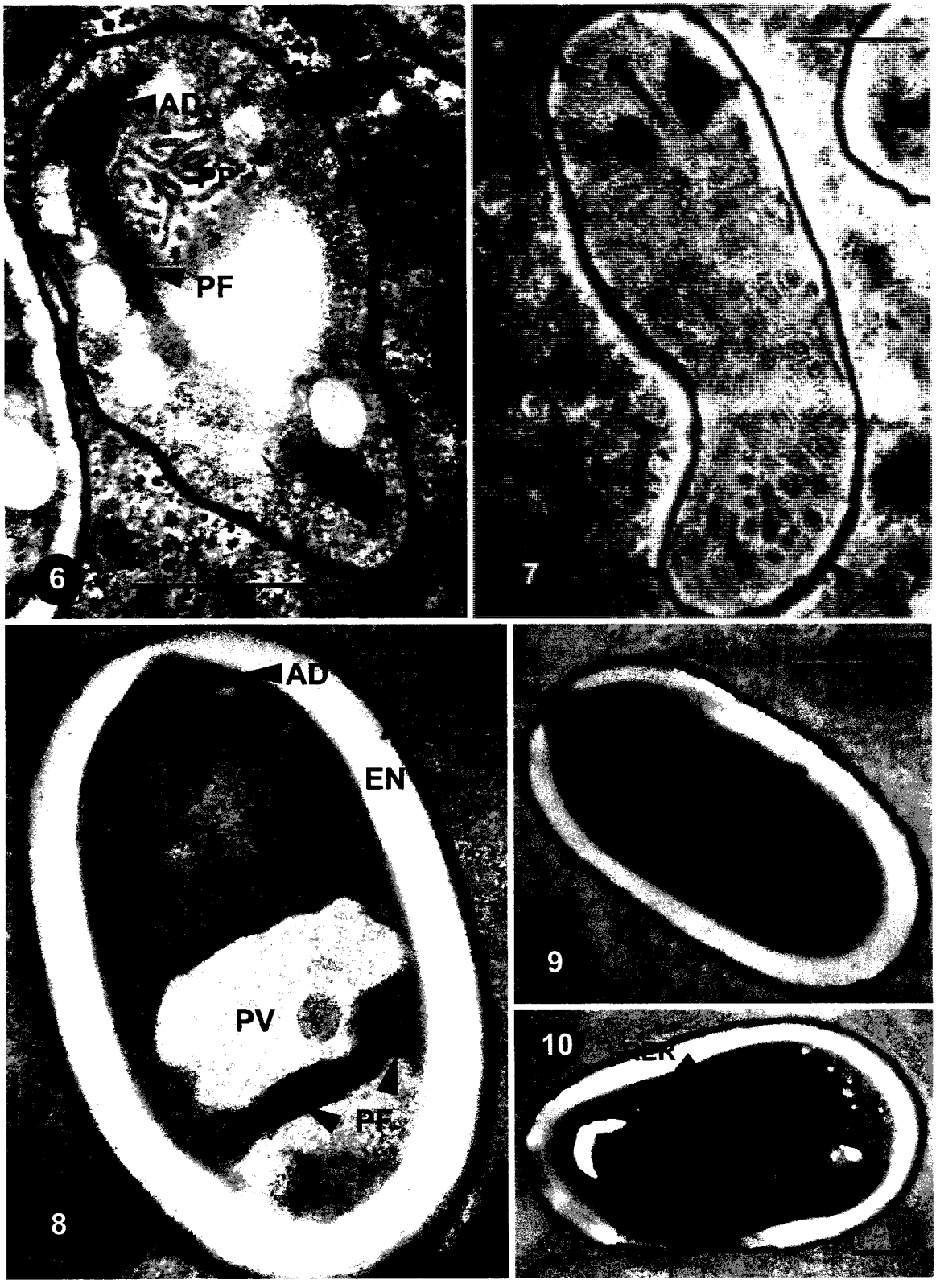
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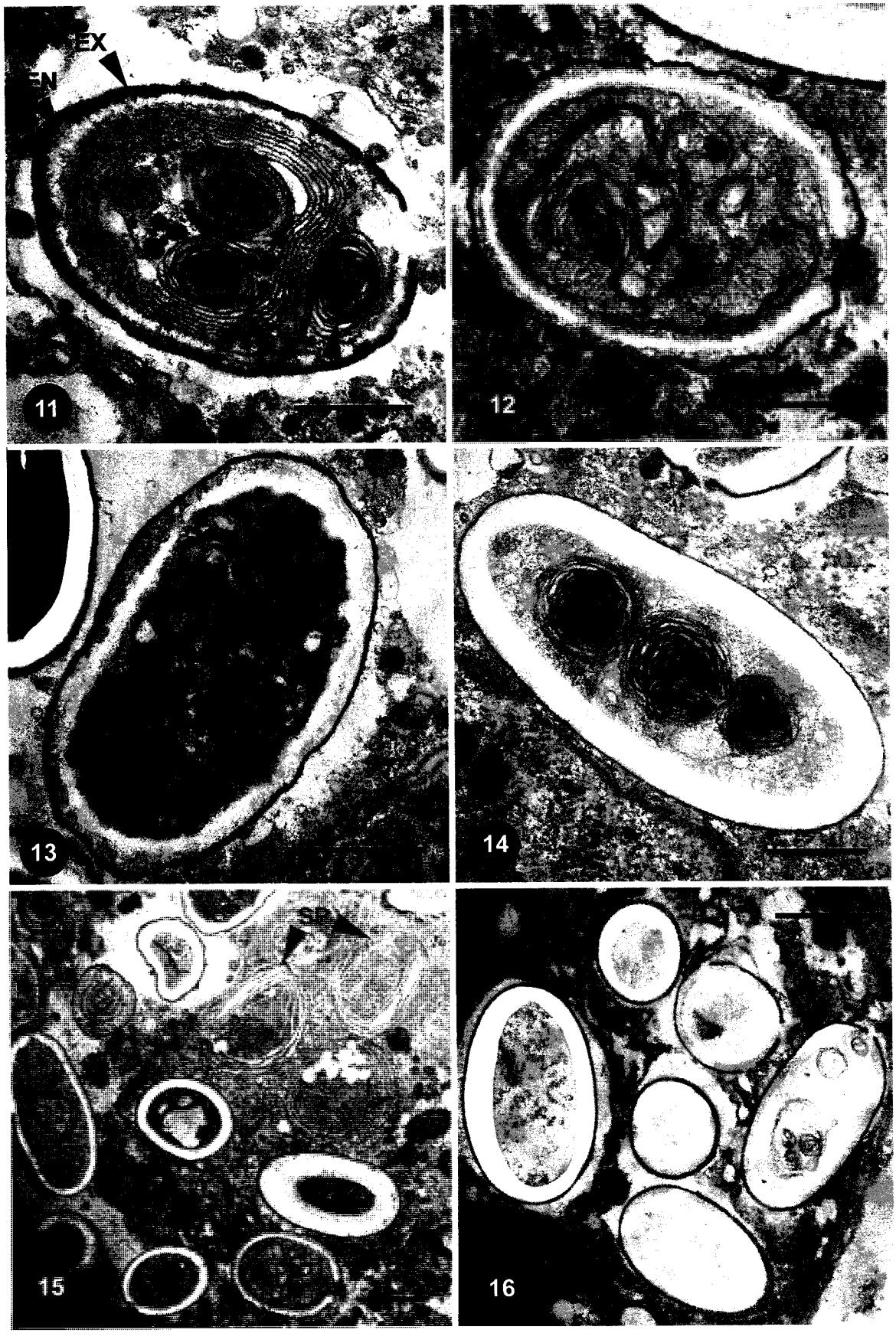
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Appendix

Tissue Preparation and Embedment Protocol for Transmission Electron Microscopy (Becnel, 1997)

FIXATION:

1) Dissect specimen in 2.5% glutaraldehyde (5-15 mins)

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

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

4) Post-fixation:
   a) 1.0% OsO_4 (pH 7.5) – 105 -120 min, room temperature (1h45min to 2hrs)
   (vials should be wrapped in foil)

5) Double Distilled washes (3 washes, 15min each)

DEHYDRATION:

1) 10% ETOH (10mins)
2) 30% ETOH (10mins)
3) 50% ETOH (10mins)
4) 70% ETOH (10mins, note: a good point for en-block staining, wrap in foil overnight)
5) 80% ETOH (10mins)
6) 90% ETOH (10mins)
7) 95% ETOH (10mins)
8) 100% ETOH (2 washes, 15min each)
9) 100% acetone (2 washes, 15min each)

EMBEDDING:

1) resin + acetone (1:3, 2h to overnight)
2) resin + acetone (1:1, 4h)
3) resin + acetone (3:1, 4h)
4) 100% resin (4h to overnight) while under vacuum (15 psi)
5) Pure resin (change vials), all day (6 hours)
6) Embed in capsules that have dried for at least 24hours in 60°C oven
   - add small drop of fresh plastic into tip of capsule
   - place tissue into the drop and fill the 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

Performed immediately prior to viewing
1) Float grids onto a drop of uranyl acetate (5 mins)
2) Pass grids through de-ionized water 3 times, blot and immediately submerge into a drop of lead citrate, section side up (5 mins)
3) Submerge in de-ionized water, and allow to air dry

Semi-thin section staining:
1) Semi-thin sections placed onto glass slides and heated on hotplate
2) Toluene blue was applied to glass sections and allowed the evaporate
3) Methanol used to wash excess
4) Apply permount and coverslip, allow to dry and view