Chapter 7

Microsporidian Entomopathogens

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Chapter Outline

SUMMARY

Microsporidia, pathogenic protists related to the Fungi, are considered to be primary pathogens of many aquatic and terrestrial insect species and have important roles in insect population dynamics, managed insect disease, and biological control of insect pests. Hosts are infected when spores are ingested and/or by transmission via the eggs. When ingested, spores germinate in a unique fashion: a polar tube that is coiled within the spore rapidly everts and punctures the host midgut cells, injecting the spore contents into the cell cytoplasm. Mitochondria and Golgi bodies are lacking in these obligate intracellular pathogens, and energy is evidently extracted from host cells via direct uptake of ATP. Effects on the host are typically chronic; therefore, the use of microsporidia in biological control programs focuses on inoculative introductions, augmentative release, and conservation biology. This chapter reviews the biology, ecology, pathology, and classification of microsporidia with examples of several long-term research efforts to manipulate these pathogens for the suppression of insect pests.

7.1. INTRODUCTION

"Enigmatic", in the words of Maddox and Sprenkel (1978) and echoed by succeeding generations of researchers (Canning *et al.*, 1985; Bigliardi, 2001; Susko *et al.*, 2004;

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James *et al.*, 2006), aptly describes the group of fascinating but elusive pathogens comprising the phylum Microsporidia. This particular label remains a not-so-subtle indication of the difficulties encountered in studies of these organisms and the still perplexing phylogenetic relationships at all taxonomic levels. Nevertheless, it is important to consider the role of the microsporidia in natural systems and in biological control programs because they are nearly ubiquitous pathogens of insects and other arthropods. Most of the more than 1300 described species in approximately 186 genera are pathogens of invertebrate animals, with insects being type hosts of nearly half of described genera (Becnel and Andreadis, 1999). Close inspection of most insect taxa yields new species (Brooks, 1974). All microsporidia are obligate intracellular pathogens of protists and animals, including both warm-blooded and cold-blooded vertebrates. They do not reproduce as free-living organisms and they are not plant pathogens.

Microsporidia are important primary pathogens of both pest and beneficial insects. The first known microsporidium, *Nosema bombycis*, was described from the silkworm, *Bombyx mori*, by Nägeli (1857), who considered the pathogen to be a yeast. Symptoms caused by *N. bombycis* infection, such as dark spots on the larval integument, were first noted by Jean Louis Armand de Quatrefages in the early 1800s and named "pébrine" (pepper) disease. Louis Pasteur's studies of pébrine disease (Pasteur, 1870) provided methods of prevention and control and were credited with saving the silkworm industry in France (see Chapters 2 and 12).

The silkworm was the first beneficial insect noted to be devastated by microsporidia, but several microsporidian species have serious impacts on other managed insects that are reared in high-density colonies, as well as on natural populations of beneficial insects (Chapter 12). Two microsporidian species, *Nosema apis* and *N. ceranae*, are pathogens of honey bees; *N. ceranae* has been implicated as a contributing factor in the decline of managed bees (reviewed by Paxton, 2010), although effects of the disease reported in the literature are highly variable. *Nosema bombi* is a Holarctic generalist pathogen of bumble bees (*Bombus* spp.). High prevalence levels in some *Bombus* species that are apparently in decline have led to questions about the possible introduction of virulent strains and the potential role these pathogens play in permanently reducing *Bombus* spp. range and population numbers in North America (Thorp and Shepherd, 2005; Cameron *et al.*, 2011).

Microsporidia are also important as regulatory factors in populations of insect pests. Hajek *et al.* (2005) listed five microsporidian species that have been introduced as biological control agents globally. They include *Paranosema* (*Nosema*) *locustae* used as a microbial insecticide for grasshoppers and established in Argentina in several orthopteran species (see Section 7.4.4); *Vavraia* (*Pleistophora*) *culicis* introduced into *Culex* mosquito populations in Nigeria, where establishment was not confirmed; *Nosema pyrausta*, a pathogen of the European corn borer, *Ostrinia nubilalis*, introduced from Iowa as an augmentative control in Illinois *O. nubilalis* populations, and persisting in the Illinois populations; and the European gypsy moth (*Lymantria dispar*) pathogens *Nosema portugal* and *Endoreticulatus* sp. (*E. schubergi*, = *Vavraia* sp., = *pleistophora schubergi* introduced into an isolated gypsy moth population in Maryland. Persistence of *N. portugal* was confirmed for one year (see Section 7.4.6). While probably lacking utility as microbial insecticides, these typically chronic pathogens clearly have a role as major components of the natural enemy complex of many insect species (Kohler and Wiley, 1992; Maddox, 1994; Lewis *et al.*, 2009) and warrant continued study for their use in classical, augmentative and conservation biological control programs.

7.2. CLASSIFICATION AND PHYLOGENY

Only in the past 15 years have microsporidian genes been analyzed to the extent that placement of this eukaryotic pathogen group in the Protozoa by Balbiani (1882) was seriously questioned. Recent molecular studies placed the phylum Microsporidia within the kingdom Fungi (Keeling and Doolittle, 1996; Hirt *et al.*, 1999; Bouzat *et al.*, 2000; James *et al.*, 2006; Hibbett *et al.*, 2007), with evidence, albeit controversial, that the group is related to the entomopathogenic Zygomycetes (Keeling, 2003; Lee *et al.*, 2008; Corradi and Slamovits, 2010). Koestler and Ebersberger (2011) point out that although Microsporidia and Zygomycetes alone among the fungi share a three-gene cluster encoding a sugar transporter, a specific transcription factor, and RNA helicase, clustering of these genes appears to be ancestral in eukaryotes and is, therefore, not phylogenetically informative. Questions about the phylogenetic placement of microsporidia thus remain without definitive answers. Although the predominance of evidence places microsporidia within the fungal lineage (Corradi and Keeling, 2009), their morphology, biology, and host interactions are unique. In this chapter, the microsporidia are treated as a separate group of insect pathogens.

The first modern classification for the microsporidia was proposed by Tuzet *et al.* (1971) and since that time, the classification of the microsporidia at all taxonomic levels has been controversial (reviews by Sprague, 1977a; Sprague *et al.*, 1992; Tanada and Kaya, 1993; Franzen, 2008). The classical morphological approach (based on nuclear arrangement, spore shape, structure and size, nature of vesicles, etc.) and molecular-based methods have produced conflicting interpretations (Vossbrinck and Debrunner-Vossbrinck, 2005; Larsson, 2005). A

phylogenetic tree was created using rDNA data from 125 species of microsporidia from vertebrate and invertebrate hosts (Vossbrinck and Debrunner-Vossbrinck, 2005). Five major clades were identified, and three major groups based on host habitat (freshwater, marine, and terrestrial) were suggested as being more consistent with evolutionary relationships than previous higher level classifications based on morphology and life cycle characteristics. Nevertheless, there were numerous examples of species that did not fit into the expected grouping, such as some true *Nosema* species (type host from Lepidoptera) reported from aquatic crustaceans.

Like habitat, host taxon is not always a reliable basis for microsporidian classification. Examples include the genus *Encephalitozoon*, for which all known species are pathogens of vertebrate animals with the exception of one species isolated from *Romalea microptera*, a lubber grasshopper (Lange *et al.*, 2009). Ribosomal RNA sequences from a crayfish microsporidium placed it in the genus *Vairimorpha* (Moodie *et al.*, 2003), previously described only from Lepidoptera and two other insect orders. In other genera, similar morphological characters among species that were isolated from unrelated host species have probably obscured true phylogenetic relationships. According to Vossbrinck and Debrunner-Vossbrinck (2005), "Molecular phylogenetic analysis has revealed that genera such as *Nosema*, *Vairimorpha*, *Amblyospora*, *Thelohania* and *Pleistophora* are polyphyletic in origin and efforts are being made to reclassify species unrelated to the type species." There are many microsporidian species that will need to be reassigned to new genera and families. Determining the critical cytological and genetic features is the next challenge in establishing a sound taxonomic system for the microsporidia.

7.2.1. Overview of Microsporidian Entomopathogens in a Phylogenetic Context

Insects are represented as type hosts of 90 microsporidian genera, nearly half of all those described (Table 7.1). Microsporidia isolated from insects have also been assigned to genera with type species from hosts in other taxonomic classes, and microsporidia from non-insect hosts have been assigned to genera with an insect type species. In both cases, additional information, primarily at the molecular level, will determine whether the generic assignments are valid with respect to the type species, and will perhaps clarify genera that may be restricted to a particular host group and those that have a broad host range. Examples of two microsporidian clades that represent a large portion of insect microsporidia are presented here.

Amblyospora/Parathelohania *Clade*

The *Amblyospora/Parathelohania* clade contains more than 122 of the approximately 150 species of microsporidia that have been described from mosquito hosts (*Amblyospora* more than 100 species, *Parathelohania* about 22 species), with at least 21 other genera reported from mosquitoes but none with more than two species (Andreadis, 2007). The *Amblyospora/Parathelohania* clade is therefore a very important and common group of microsporidia notable for a number of reasons. First, it is the most widely observed group of microsporidian pathogens in larval mosquitoes because the infections are easily recognized by the presence of large white cysts filled with spores located in the fat body. Second, *Amblyospora* and *Parathelohania* species are representative of polymorphic microsporidia (more than one sporulation sequence) and were the first species for which an intermediate copepod host was found to be involved in the life cycle (Becnel and Andreadis, 1999; Andreadis, 2007). The complex life cycles include alternations of haploid and diploid cell states (which usually involves meiosis) as well as horizontal and vertical (transovarial) transmission involving larval and adult mosquitoes and copepods. Members of this clade are usually host specific, with *Amblyospora* spp. mainly restricted to *Aedes/Culex* mosquitoes and *Parathelohania* spp. to *Anopheles* spp.

Small subunit ribosomal DNA sequences from a large number of species in the *Amblyospora/Parathelohania* clade have permitted an in-depth analysis of the phylogenetic relationships among the microsporidia, as well as the relationships to the host groups. The *Amblyospora/Parathelohania* and *Amblyospora/Parathelohania*-like genera form a very strongly supported clade, demonstrating the close relationship among these polymorphic microsporidia in mosquitoes (Vossbrinck *et al.*, 2004; Vossbrinck and Debrunner-Vossbrinck, 2005). Analysis has also shown a possible evolutionary correlation between *Aedes* and *Culex* hosts with *Amblyospora* spp. and between *Anopheles* hosts with *Parathelohania* spp. (Baker *et al.*, 1998; Vossbrinck *et al.*, 2004). Not all species within this clade involve an intermediate host (notably *Edhazardia aedis* and *Culicospora magna*) but otherwise have complex life cycles that include both horizontal and vertical transmission. Studies of species within the *Amblyospora/Parathelohania* clade have made significant contributions to an overall understanding of microsporidian biology relative to developmental sequences and complete life cycles. As additional genomic data are generated on this and other groups of microsporidia, a better understanding should emerge of phylogenetic relationships, as well as information on sexuality, host range determinants, and evasion of host immune response, among many other basic biological features.

TABLE 7.1 Genera of microsporidian type species isolated from insects; listed by insect host taxonomic order.

Insect Order	Microsporidian Genus	Insect Order	Microsporidian Genus
Collembola	*Auraspora*	Diptera	*Tricornia*
Diptera	*Aedispora*		*Tubilinosema*
	Amblyospora		*Vavraia*
	Andreanna		*Weiseria*
	Anisofilariata		*Pegmatheca*
	Bohuslavia		*Pernicivesicula*
	Campanulospora		*Pilosporella*
	Caudospora		*Polydispyrenia*
	Chapmanium		*Ringueletium*
	Coccospora		*Scipionospora*
	Crepidulospora		*Semenovaia*
	Crispospora		*Senoma*
	Cristulospora		*Simuliospora*
	Culicospora		*Spherospora*
	Culicosporella		*Spiroglugea*
	Cylindrospora		*Striatospora*
	Dimeiospora		*Systenostrema*
	Edhazardia		*Tabanispora*
	Evlachovaia		*Toxoglugea*
	Flabelliforma		*Toxospora*
	Golbergia		*Trichoctosporea*
	Hazardia	Coleoptera	*Anncaliia*
	Helmichia		*Cannngia*
	Hessea		*Chytridiopsis*
	Hirsutusporos		*Endoreticulatus*
	Hyalinocysta		*Ovavesicula*
	Intrapredatorus	Ephemeroptera	*Mitoplistophora*
	Janacekia		*Pankovaia*
	Krishtalia		*Stempellia*
	Merocinta		*Telomyxa*
	Napamichum		*Trichoduboscqia*
	Neoperezia	Hemiptera	*Becnelia*
	Octosporea	Hymenoptera	*Antonospora*
	Octotetraspora		*Burenella*
	Parapleistophora		*Kneallhazia*
	Parastempellia	Isoptera	*Duboscqia*
	Parathelohania		

TABLE 7.1 Genera of microsporidian type species isolated from insects; listed by insect host taxonomic order—cont'd

Insect Order	Microsporidian Genus	Insect Order	Microsporidian Genera Totals
Lepidoptera	*Cystosporogenes*	Collembola	1
	Larssoniella	Diptera	57
	Nosema	Coleoptera	5
	Orthosomella	Ephemeroptera	5
	Vairimorpha	Hemiptera	1
Odonata	*Nudispora*	Hymenoptera	3
	Resiomeria	Isoptera	1
Orthoptera	*Heterovesicula*	Lepidoptera	5
	Johenrea	Odonata	2
	Liebermannia	Orthoptera	4
	Paranosema	Siphonaptera	2
Siphonaptera	*Nolleria*	Thysanura	1
	Pulicispora	Trichoptera	3
Thysanura	*Buxtehudea*		
Trichoptera	*Episeptum*	**Total**	**90**
	Issia		
	Tardivesicula		

Nosema/Vairimorpha *Clade*

Microsporidia in the *Nosema/Vairimorpha* clade are common and important pathogens of insects with terrestrial life cycles and are particularly well represented in Lepidoptera. The type microsporidian species, *Nosema bombycis*, is currently described (formally and informally) on the basis of having one mature infective spore type (*N. bombycis* is now known to produce a second spore type, an internally infective spore; see Section 7.2.2), no pansporoblastic membrane, diplokaryotic nuclei throughout the life cycle, and one sporont giving rise to two mature spores by binary fission. This relatively simple life cycle, or recognizable stages of it, resulted in well over 200 species descriptions and an additional 50 descriptions to the genus level (Sprague, 1977a). The genus *Vairimorpha*, several species of which were considered to be mixed infections of *Nosema* and *Thelohania* species (e.g., *Nosema lymantriae* + *Thelohania similis* in the gypsy moth; *Nosema necatrix* + *Thelohania diazoma* in noctuid species), was recognized in the 1970s to be a polymorphic taxon with *Nosema*-like dikaryotic spores and *Thelohania*-like monokaryotic octospores (Maddox and Sprenkel, 1978), and a new genus was erected (Pilley, 1976). Molecular data subsequently identified the close relationship of the monomorphic *Nosema* and polymorphic *Vairimorpha* (Baker *et al.*, 1994; Vossbrinck and Debrunner-Vossbrinck, 2005). The octospores are ancestral sexual meiospores that have been lost multiple times within the *Nosema/Vairimorpha* clade (Ironside, 2007), leaving approximately 10 *Vairimorpha*-type species represented within most closely related species groups of the *Nosema* clade. In addition to loss of meiospores, loss of diplokarya may have occurred in the *Nosema/Vairimorpha* clade. *Oligosporidium occidentalis*, a monokaryotic (or haplokarotic) species isolated from predatory mites (Becnel *et al.*, 2002), is a sister species to *N. bombi* (Vossbrinck and Debrunner-Vossbrinck, 2005).

Species in the *Nosema/Vairimorpha* group are primarily pathogens of Lepidoptera but have also been isolated from hosts in other insect orders. For example, species from Hymenoptera include *Nosema vespula* (wasps), *N. ceranae* and *N. apis* (honey bees), *N. bombi* (bumble bees), and *Vairimorpha invictae* (fire ants), as well as from other classes of arthropods including *Nosema granulosis* (copepods) and *V. cheracis* (crayfish). The genus *Nosema* has, however, long been recognized as

a "catch-all" group for microsporidia with similar life cycles, and a large number of species has recently been transferred to other genera based on molecular characters. Additional genetic information should lead to a new characterization for the genus *Nosema* or possible new genera for the larger clade.

Species descriptions for the phylum Microsporidia are currently governed by the International Code of Zoological Nomenclature. Microsporidian taxonomists, mycologists, and other researchers agree that, although the Fungi are classified under the Code of Botanical Nomenclature, the Microsporidia should remain with the zoological code (Weiss, 2005; Redhead *et al.*, 2009).

7.2.2. General Characteristics of Microsporidia

Microsporidia, as obligate intracellular pathogens, utilize host tissues for reproductive energy and development. Organelles that are typically found in eukaryotic organisms, including peroxisomes, vesicular Golgi membranes, and mitochondria, are lacking in microsporidia. Thin Golgi-like tubules transport proteins from the endoplasmic reticulum, but no Golgi vesicles form (Beznoussenko *et al.*, 2007). Vestigial mitochondria are present in the form of mitosomes that may retain some capability for metabolic import (Williams and Keeling, 2005; Burri *et al.*, 2006). At a minimum, the microsporidia produce two or three reproductive vegetative stages (described below in Sections 7.2.2 and 7.3.1), and a mature, environmentally resistant, infective spore or "environmental spore" (Maddox *et al.*, 1999), although species exist for which no presporulation stages have been observed (Larsson, 1993). Additional characteristics that are detailed in this chapter include: (1) spore types, of which there may be one to four per species; (2) tissue tropism that ranges from utilization of one or a few host tissues to systemic infections; (3) effects on infected hosts, which vary from apparently benign to highly virulent; (4) transmission, which most typically occurs via oral ingestion of infective spores, infected female to offspring, or by both mechanisms; and (5) host specificity, ranging from highly host specific species to generalists, usually within an insect order; however, parasitoids and intermediate hosts in other orders or classes may also become infected.

Morphology

Environmental spores are readily observed in infected tissues with a light microscope ($\geq 250\times$) and are distinguished by a relatively featureless and smooth rounded surface (Fig. 7.1A) that results in birefringence under phase-contrast microscopy (Tiner, 1988). Mature spores possess a dense spore wall composed of a proteinaceous exospore and alpha-chitin and protein endospore layers (Vávra and Larsson, 1999; Y. Xu *et al.*, 2006), and are evenly bright in appearance. The endospore in aquatic species tends to be thinner than that of terrestrial species [compare transmission electron micrographs of mature microsporidian spores from aquatic hosts (Terry *et al.*, 1999; Micieli *et al.*, 2003; Nylund *et al.*, 2010) with those isolated from terrestrial hosts (Vávra *et al.*, 2006; Wang *et al.*, 2009; Sokolova *et al.*, 2010)], and the distal vacuole or spore contents may be vaguely visible. The general spore shapes, frequently oval, long oval, or egg, spindle, or tear shaped, are consistent within each sporulation sequence for a species, but there is much overlap among species and shape alone cannot be used for species identification. Spore length, also relatively consistent within a species (usually within 0.5 μm), ranges from 1.5 to 10 μm, with 2−6 μm being most common in insect hosts. Giemsa-stained environmental spores (Fig. 7.1B) appear white with blue-tinted spore walls and generally show a characteristic blue stain on the surface. Spores appearing gray under phase-contrast microscopy may be either immature or inviable (Fig. 7.1A) and, if recently germinated, may appear to be a "shell" with a gray interior and strongly visible spore walls (Fig. 7.1C). Some microsporidian species produce a "primary" spore, an internally infective stage that possesses a thin endospore and organelles that appear to be less well developed. Typical of the genus *Nosema* as well as some other genera, the primary spores are less refringent than environmental spores and may appear slightly more rounded in shape with a visible polar vacuole (Fig. 7.1C). Vegetative (reproducing) forms are difficult to detect under light microscopy, but when infections are intense, they may be observed as small (typically 2−10 μm), round cells with smooth cytoplasm and visible nuclei (Fig. 7.1D). Reproduction in some species entails a series of nuclear divisions that produce vegetative cells with multiple nuclei called plasmodia. These cells may be round (Fig. 7.2) or ribbon like.

The intracellular features of microsporidia are clearly discernible only in high-magnification transmission electron micrographs. Each environmental spore contains either a single nucleus (monokaryon) or a double nucleus (diplokaryon), depending on species. Some species possess both spore types over the course of the life cycle; for example, the terrestrial *Vairimorpha* species produce diplokaryotic spores and monokaryotic meiospores that are formed in a vesicle of eight spores (Moore and Brooks, 1992; Vávra *et al.*, 2006). The aquatic *Amblyospora* and *Edhazardia* species also produce both diplokaryotic and monokaryotic spores (Becnel, 1992a, 1994) (Fig. 7.3). The diplokaryotic form in those species that possess this configuration is considered to be genetically diploid. Vegetative forms may differ in number of nuclei from one to many, depending on the stage of development; whether monokaryotic or dikaryotic or developing as plasmodia

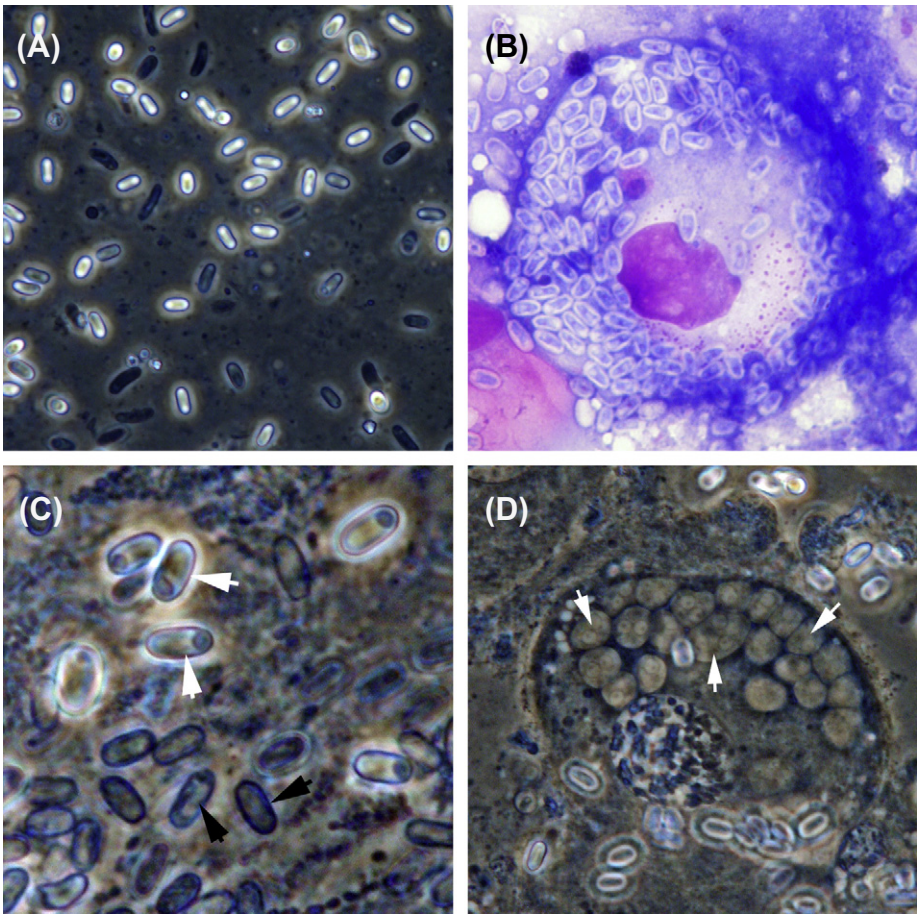


FIGURE 7.1 Phase-contrast micrographs of life cycle stages of a *Nosema*-like microsporidium isolated from black vine weevil, *Otiorynchus sulcatus*. (A) Environmental (brightly refractive) spores and immature (dark gray) spores (1000 ×); mature microsporidian spores are typically smooth and birefringent under phase-contrast microscopy. Immature spores lack a developed endospore and appear gray. (B) Giemsa stained environmental spores in a midgut epithelial cell (500 ×). (C) Group of primary spores (white arrows) and germinated primary spores (black arrows) in midgut epithelia (1000 ×). (D) Vegetative forms (arrows), in midgut epithelial cell (1000 ×). *(Photos by L. F. Solter.)*

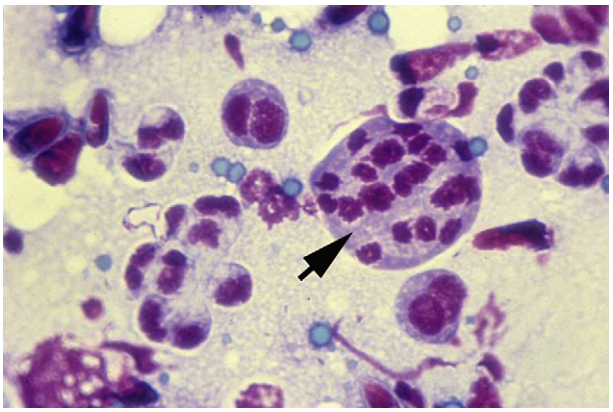


FIGURE 7.2 Giemsa stain of *Culicosporella lunata* vegetative plasmodium (black arrow). *(Photo by J. J. Becnel.)*

(Fig. 7.2); and whether division is by binary fission or multiple fission/budding.

Microsporidia possess uniquely identifying ultrastructural features (Fig. 7.4). A plate or anchoring disk at the anterior end of the spore attaches a specialized organelle, the polar filament, that coils around the interior of the spore. This flagellum-like filament is everted to become the polar tube through which the spore contents are injected into a host cell (Frixione *et al.*, 1992). Polar tubes occasionally can be observed under light microscopy immediately after the spores have germinated. This structure is unique to the Microsporidia and defines the taxon (Sprague, 1977a). Layers of membranes, the polaroplast, at the anterior end of the spore appear to be involved in the germination process and may serve as the plasmalemma of the new vegetative stage (germ) infecting the host cell (Weidner *et al.*, 1984). The posterior of the spore is occupied by a posterior vacuole that appears to be involved in spore germination (Findley *et al.*, 2005). The fine structure of microsporidia is presented in a detailed review by Vávra and Larsson (1999), and the reader is referred to this excellent text for more detailed information.

Genetic Characters

Information about the microsporidian genome is changing rapidly with increasingly sensitive and affordable technology. The first sequenced genome, *Encephalitozoon cuniculi* (Katinka *et al.*, 2001), provided information about one of the

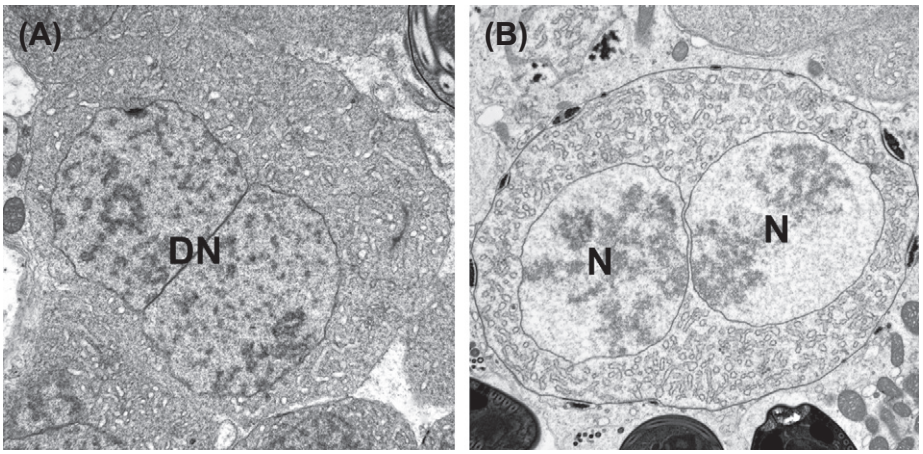


FIGURE 7.3 Transmission electron micrographs of (A) *Culicosporella lunata* diplokaryon; (B) a meiotic division in progress that will terminate in two cells, each monokaryotic. DN = diplokaryotic nucleus; N = nucleus. *(Photos by J. J. Becnel.)*

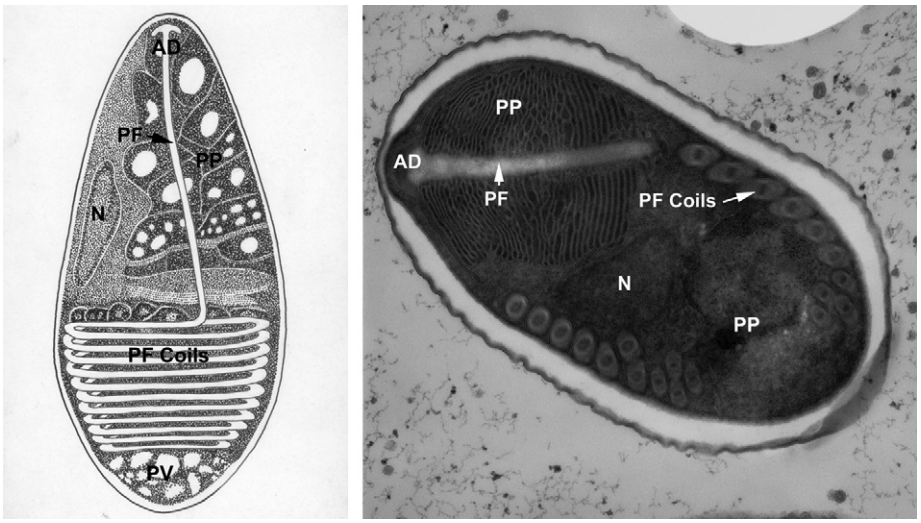


FIGURE 7.4 Schematic (left) and transmission electron micrograph (right) of an environmental spore. AD = anchoring disk; PP = polaroplast; PF = polar filament; N = nucleus; PV = posterior vacuole. *(Drawing and photo by J. J. Becnel.)*

most reduced and compact genomes known in eukaryotes at 2.9 million base pairs (Mbp) (Keeling *et al.*, 2005), and was notable for short intergenic sequences, only 2000 protein-encoding genes, and its lack of repeats and transposable elements (Keeling, 2009). With more genomes currently sequenced and at least partially analyzed, the genome architecture has been shown to vary considerably across the phylum. Insect pathogenic species apparently possess numerous transposable elements and genome sizes that range from about 7 to 20 Mbp (J. Xu *et al.*, 2006; Williams *et al.*, 2008b; Keeling, 2009; Texier *et al.*, 2010). One grasshopper species, *Encephalitozoon romaleae*, and its human pathogen congener, *Encephalitozoon hellem*, were shown to possess protein-encoding gene sequences of insect origin in the genome (Selman *et al.*, 2011).

Microsporidian species are currently described based on a combination of morphology, host species, and the small subunit ribosomal RNA gene (SSU rRNA) sequence of approximately 1200 base pairs (Weiss and Vossbrinck, 1999). The gene is, however, highly conserved and may vary little among closely related species (Pieniazek *et al.*, 1996; Pomport-Castillon *et al.*, 1997; Vávra *et al.*, 2006). The internal transcribed spacer (ITS) region, with flanking portions of the SSU and the large subunit (LSU), is also used to evaluate close taxonomic relationships, but polymorphisms in the ITS region as well as in the SSU of some species can obscure analysis. Other genes such as alpha-tubulin, beta-tubulin, and heat shock protein (HSP-70) have been used for taxonomic purposes, but are also highly conserved.

7.3. LIFE HISTORY

While there are unifying characteristics of infection by microsporidia, life cycles range from the very simple, involving oral transmission and reproduction of vegetative forms in host tissues to form infective environmentally resistant spores, to complex cycles that entail production of two to four spore types and involve intermediate hosts. Pathologies range from relatively slight, allowing the host to develop and reproduce, to severe, resulting in early mortality in a large percentage of infected hosts. Tissue tropism and transmission mechanisms also vary among species.

7.3.1. Infection and Replication

Microsporidia typically infect a susceptible host when spores are ingested during feeding. The current understanding is that the spores are activated by constituents of the host gut environment, possibly pH, ions, or a combination of these, as well as other conditions that the pathogen recognizes as "host" (reviewed by Keohane and Weiss, 1998). Although some species are probably activated to germinate by direct contact with host cell membranes (Magaud *et al.*, 1997; Xu *et al.*, 2003; Hayman *et al.*, 2005; Southern *et al.*, 2006), most studies evaluated human pathogens in the genus *Encephalitozoon* that do not need to breach a peritrophic membrane in the host alimentary track. Germination of the entomopathogen *N. ceranae* occurred with no contact between spores and host cells in tissue culture (Gisder *et al.*, 2011). During the germination process, the cell swells with water, causing the polar filament to evert, rapidly extend from the spore and puncture a host cell. The spore contents, including the nucleus, membranes, and other cellular constituents, move through the polar tube and are injected into the cytoplasm of the host cell.

Obligate parasitism has resulted in the loss of functioning mitochondria (Williams *et al.*, 2002; Williams and Keeling, 2005) and recent research suggests that microsporidia probably import adenosine triphosphate (ATP) directly from host cells (Weidner and Trager, 1973; Bonafonte *et al.*, 2001; Williams *et al.*, 2008a; Keeling *et al.*, 2010) as an energy source. The type of nutrititive resources taken from the host cell environment is not specifically known, but the *E. cuniculi* genome encodes several transporters that may be involved with absorption of sugars and ions (Katinka *et al.*, 2001), consistent with carbohydrate depletion in gypsy moth larvae observed by Hoch *et al.* (2002).

Vegetative division begins as early as 30 min postinoculation (Takvorian *et al.*, 2005) by binary fission (mitotic merogony), or by production of many nuclei without cytokinesis to form multinucleate plasmodia, depending on species. Plasmodia undergo multiple fission to produce daughter cells that can continue in merogony or enter sporogony. Sporulation, sporogony followed by spore morphogenesis, is the process of spore formation from a late vegetative form called the sporont, which then produces sporoblasts that mature into infective spores. Budding in the manner of yeasts has been observed in only one species, *Chytridiopsis typographi*, a pathogen of the bark beetle *Ips typographus* (Tonka *et al.*, 2010). Movement within the host tissues from cell to cell is not well understood, but some species form primary spores that germinate within the tissues, apparently to infect adjacent cells and tissues. This cycle occurs in several microsporidian genera including those infecting terrestrial insect hosts (Iwano and Ishihara, 1989; Fries *et al.*, 1992; Solter and Maddox, 1998a) and aquatic insect hosts (Johnson *et al.*, 1997). Phagocytosis by host cells and subsequent germination of spores held within the phagosomes may also be involved (Takvorian *et al.*, 2005).

Reproduction in most species of microsporidia was assumed to be clonal (evolution from ancient asexual organisms), but recent genetic studies suggest that sexual genetic exchange may occur during vegetative reproduction (Sagastume, 2011). In addition, spore forms that are evidently the end-products of meiotic divisions are found in some species, e.g., the terrestrial *Vairimorpha* species and aquatic *Amblyospora* species, while other closely related species have no obvious sexual forms, suggesting that microsporidia evolved from sexual ancestors but have lost sexuality multiple times (Ironside, 2007).

7.3.2. Pathology

Microsporidian infections tend to be chronic in nature, but effects range from nearly benign to relatively virulent, with infections causing death of the host, albeit often slowly, even when dosages are low. Typical effects are sublethal and include extended larval development period, sluggishness, lower pupal weight, and lower fecundity if the host survives to eclose and mate. Adult life span is frequently reduced and immature insects that acquire infections may not survive later molts or pupation.

Microsporidia may invade one or a few tissues, typically the alimentary tract or fat body, or may cause systemic infections. Tissue tropism is a species-specific character, varying among microsporidian genera and even species within genera. All known *Endoreticulatus* spp. are pathogens of the alimentary tract (Brooks *et al.*, 1988), while species in the *Nosema/Vairimorpha* clade may be restricted to the midgut (e.g., *N. apis* and *N. ceranae* in

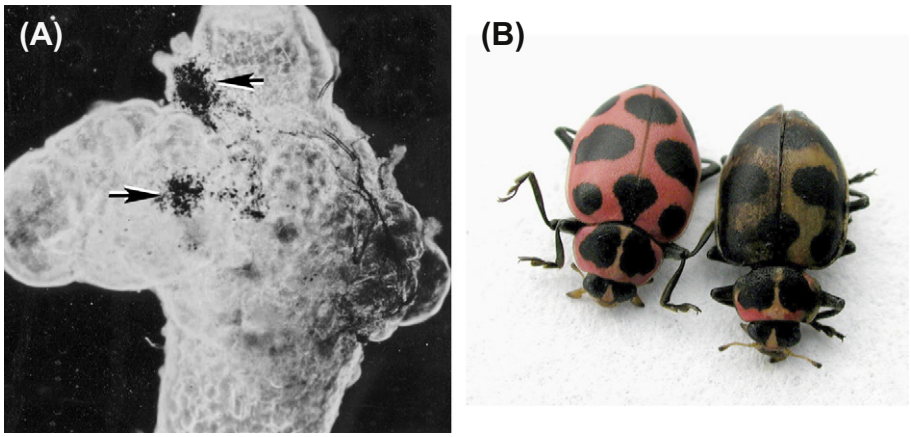


FIGURE 7.5 (A) Melanized areas (black arrows) of gastric caeca of *Aedes aegypti* infected with *Edhazardia aedis*. (B) Healthy pink lady beetle (*Coleomegilla maculata*) (left) and beetle infected with an undescribed microsporidium showing melanization (right). *(Photos by M. Rotstein (A) and S. E. White (B).)*

honey bees), primarily fat body pathogens (*Vairimorpha* spp.) or systemic (*N. bombycis* and closely related "true *Nosema*" species).

Tissue trophism of a well-studied microsporidian species in mosquitoes involves sequential infection of specific tissues, with initial infections in the larval alimentary tract followed by infection of larval oenocyes that are carried into the adult mosquito. These oenocytes invade the ovaries of adult females where binucleate spores are formed that germinate and infect the developing eggs. Sexual dimorphism in the progeny of some mosquito species results in fat body infections in male larvae causing death and release of meiospores, while infections in female larvae are benign and produce infected adults to complete the life cycle. The meiospores infect a copepod intermediate host, which produces spores that are infectious to mosquito larvae upon ingestion (Becnel and Andreadis, 1999; Andreadis, 2007).

Microsporidia that are systemic tend to produce fewer spores per unit of infected tissue and are, therefore, often less virulent than species that specifically target fat body tissues. Lower virulence may allow the host to complete its life cycle and, for many species, ensures transmission of the pathogen to the next host generation via the egg or embryo (Becnel and Johnson, 2000; Dunn *et al.*, 2001; Andreadis, 2005; Haine *et al.*, 2005). Fat body pathogens, for example, the lepidopteran *Vairimorpha* species, may result in nearly 100% mortality of larvae at any infective dosage (Goertz and Hoch, 2008a). The fate of immature insects infected with many other species of microsporidia may depend on number and viability of spores consumed, age and stage at exposure, and additional external stress. Insects infected as embryos often have high mortality rates (Andreadis, 1986; Han and Watanabe, 1988)

Microsporidia primarily develop in the cytoplasm of host cells, but several species infecting fish mature in host cell nuclei (Lom and Dykova, 2002; Nylund *et al.*, 2010), as does a recently described species, *Enterospora canceri*, isolated from an arthropod, the brown crab (Stentiford *et al.*, 2007). Reports of development in host cell nuclei of insects are rare (Sprague *et al.*, 1992; Becnel and Andreadis, 1999). Vegetative reproduction, but not spore formation of *N. portugal*, was observed in the nuclei of silk gland cells of the gypsy moth host (Maddox *et al.*, 1999), and vegetative forms of the type species of several microsporidian genera develop in close contact with the host cell nucleus, sometimes in deep indentations of the nuclear membranes (Sprague *et al.*, 1992).

Many microsporidian species cause hyperplasia and/or hypertrophy of the nuclei and cytoplasm of infected cells; xenomas, fusion or extension of hypertrophic infected cells to form tumors or cysts, are rare but do occur in insects (Becnel and Andreadis, 1999). Infections may become so severe that the cells of target tissues are filled with spores, interfering with normal cellular function and often resulting in death of the host. A common immune response by insects to microsporidian infection is melanization of infected cells (Fig. 7.5A). Dark melanized areas can sometimes be observed through the epidermis of the host (Fig. 7.5B) and may result in a spotted or mottled appearance called pébrine (as discussed earlier). The only other observable symptom is a puffy appearance and light color of some immature and aquatic insects with patent fat body infections. Patent infections in tissues, however, may be easily observed when insects are dissected.

7.3.3. Transmission

Most microsporidian species are horizontally transmitted to susceptible hosts when environmental spores are disseminated in the feces or regurgitated matter of

infected hosts, possibly via silk (Jeffords *et al.*, 1987), and from decomposing infected hosts (Goertz and Hoch, 2011). Transmission by parasitoids, either by infected female wasps or by mechanical contamination when a wasp first oviposits in an infected host, then in an uninfected host, has been shown in laboratory studies (Own and Brooks, 1986; Siegel *et al.*, 1986a), but it is not known whether parasitoid transmission is important to the overall dynamics of microsporidian disease in insect populations. Horizontal transmission is likely whenever infections occur in gut tissues and Malpighian tubules; infections limited to the fat body require cannibalism, the death and decomposition of the host, or possibly vectoring by ovipositing parasitoids.

Many species of microsporidia, in addition to being horizontally transmitted, are vertically transmitted to the offspring of infected female hosts within or on the surface of the eggs. The egg chorion may be contaminated with infective spores as the egg is oviposited, or the embryos become infected during development. Both types of vertical transmission are considered to be transovum transmission; the latter special case is termed transovarial transmission. Venereal transmission via infected males is hypothesized to occur but does not appear to be common or important in disseminating the pathogen, even when male gonads are infected (Solter *et al.*, 1991; Patil *et al.*, 2002; Goertz and Hoch, 2011). For a detailed overview of transmission mechanisms see Becnel and Andreadis (1999).

The generation time, from inoculation to production of environmental spores in a newly infected host, may be as little as four days, although there may be a period of latency before spores are released from infected cells into the feces (Siegel *et al.*, 1988; Goertz *et al.*, 2007). Some species appear to have longer generation times and some may require a signal to form mature spores; for example, a host blood meal is required for the mosquito pathogen, *Amblyospora campbelli*, to sporulate (Dickson and Barr, 1990).

7.3.4. Environmental Persistence

The mature, infective microsporidian spore is the only life stage that is sufficiently environmentally resistant to survive outside the cells of living hosts. Although many terrestrial microsporidia can be stored for over 30 years in liquid nitrogen (Maddox and Solter, 1996), in general, infective spores from terrestrial hosts that are reasonably protected from ultraviolet (UV) radiation and other degradation factors, including other microbes, can probably survive for one month to one year under normal environmental conditions (Maddox, 1973, 1977; Brooks, 1980, 1988; Goertz and Hoch, 2008b). Some species of microsporidia isolated from aquatic hosts have survived for 10 years in sterile water suspensions under refrigeration in the laboratory (Oshima, 1964; Undeen and Vávra, 1997), but spores from such hosts cannot be frozen, cannot tolerate desiccation, and are also subject to environmental degradation in the aquatic environment (Becnel and Johnson, 2000). Microsporidia have adapted to the presence and absence of hosts due to seasonality or population density cycles with strategies such as maintenance in other related host species (Lange and Azzaro, 2008) and maintenance in alternate or intermediate hosts (Micieli *et al.*, 2009). *Edhazardia aedis* infections in the host *Aedes aegypti* may be nearly benign in some individuals, favoring vertical transmission, and patent in other individuals, serving to inoculate the local environment with spores for horizontal transmission among mosquito larvae (Koella *et al.*, 1998). Survival of microsporidia in diapausing host stages, such as *N. pyrausta* infections in overwintering fifth instar *O. nubilalis* (Andreadis, 1986; Siegel *et al.*, 1988) and *N. portugal* in transovarially infected eggs (Maddox *et al.*, 1999), is probably common. Although some hosts may die during diapause owing to additional physiological stress caused by infection (Andreadis, 1986), the pathogen is protected from environmental degradation in surviving hosts, ensuring survival until the next generation of hosts is available.

7.3.5. Life Cycles

The life cycles of several microsporidian species from terrestrial hosts and aquatic hosts were covered in detail by Becnel and Andreadis (1999), and are distinctive for both similarities and differences among species. A generalized life cycle for the genus *Nosema* is briefly described here, as well as life cycles for two species from mosquito hosts. Each of these has similar counterparts in other genera, but these descriptions do not cover the full range of possible sequences in microsporidian development.

Life Cycles of Microsporidia in the Nosema/Vairimorpha *Clade*

With the exception of the *Vairimorpha*-type microsporidia that appear in the *Nosema* clade, all *Nosema* species isolated from terrestrial hosts follow a similar sequence of sporulation events (Fig. 7.6A), with the major difference among species being tissue specificity. Some *Nosema* species, for example, those in the "true" *Nosema* group isolated from Lepidoptera, are systemic pathogens that, when orally ingested, first infect the midgut tissues, then invade most other tissues, or are systemic in the embryo if transovarially transmitted.

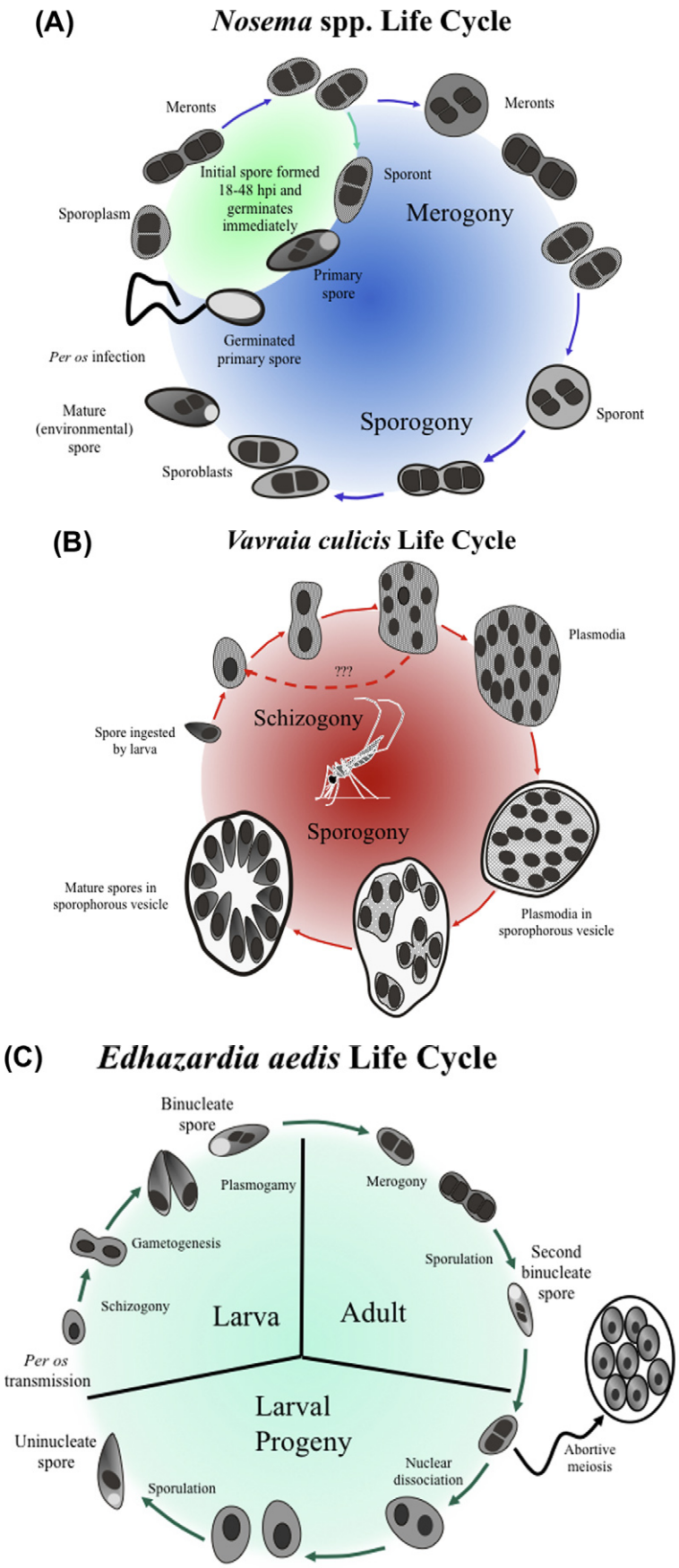


FIGURE 7.6 Life cycles of (A) *Nosema* spp. (h.p.i. = hours postinfection); (B) *Vavraia culicis*; (C) *Edhazardia aedis. (Drawings by S. E. White; courtesy of Folia Parasitologica, Institute of Parasitology.)*

Some *Nosema* species are restricted to the midgut tissues, notably, *N. apis* and *N. ceranae*, pathogens of honey bees. The gypsy moth Nosema species, *N. lymantriae* and *N. portugal*, initiate infection in the midgut epithelial cells, then primarily target the silk glands and fat body, with reproduction also occurring in the Malpighian tubules, gonads, and nerve tissues.

When *Nosema* spores are ingested, the infection cycle begins when germination occurs in the midgut lumen of the host and the sporoplasm, extruded through the polar tube, is injected into the cytoplasm of the midgut epithelial cells. The sporoplasm grows in size to become a meront, then the nucleus divides, followed by binary fission of the cell. For some species, this first division occurs between 30 mins and 12 h of infection. Monokaryotic meronts have been occasionally reported for a brief period shortly after initial infection (Maddox *et al.*, 1999; Vávra *et al.*, 2006), but it is not known whether this is an artifact of sectioning or an ephemeral phase that occurs in some species. All other stages, including those in merogonic cycles, are binucleate. Whether or not multiple merogonic divisions occur in the first cycle is probably a species character, but between 18 and 48 h postinfection, sporonts form from meronts and divide once to become two sporoblasts, each of which matures into a primary spore (described in Section 7.2.2). Primary spores germinate spontaneously, presumably infecting adjacent cells and tissues. Merogony is repeated in this second cycle and in ensuing proliferative cycles with, ultimately, development into sporonts that will divide to form thick-walled environmental spores. If the midgut tissues or Malpighian tubules are infected, environmental spores are shed in the feces of the host into the environment where they are available for ingestion by susceptible hosts.

Many *Nosema* species that produce systemic infections are also transovarially transmitted to the next host generation. These species infect the gonads of the host, and either spores or vegetative forms are incorporated into the egg during oogenesis. *Nosema granulosis*, a pathogen of the crustacean *Gamerus duebeni*, primarily infects the gonads and is apparently only transovarially transmitted. This species also feminizes its host (Terry *et al.*, 1999).

Life cycles of the *Vairimorpha* species involve a primary spore cycle in the midgut tissues and two secondary sporulation sequences in the same host: a *Nosema*-type sequence producing binucleate spores, and a sequence in which sporonts divide to form eight mononucleated spores (meiospores or, more commonly, "octospores") within a sporophorous (parasite-derived) membrane (Vávra *et al.*, 2006). With one exception, all known *Vairimorpha* species are primarily fat body pathogens and both spore types are produced in this tissue. Only the crayfish pathogen *V. cheracis* invades the muscle tissues of the host (Moodie *et al.*, 2003).

Life Cycle of Vavraia culicis

Vavraia culicis is a multisporous (multiple spores produced from plasmodia) microsporidian pathogen of *Culex pipiens* (Weiser, 1947) with all stages uninucleate (Fig. 7.6B). One type of environmental spore is produced. A sporoplasm has not been described for this species; the earliest described stage is a schizont with two or more nuclei. The plasmalemma of the schizont becomes coated with a thin, electron-dense, two-layer substance that divides when the plasmodium divides. Inside the plasmodia, nuclear divisions produce up to 16 nuclei before plasmotomy. Before sporogony, divisions occur by plasmotomy and multiple fission. Sporogony generates plasmodia that commonly produce eight, 16, or 32 nuclei. The surface layer separates from the plasmodium when sporogony is initiated, producing a sporophorous vesicle inside which lobate plasmodial stages divide to produce eight, 16, or 32 uninucleate spores. Recent evaluation of the ultrastructure and life cycles of various *Vavraia* isolates from different hosts suggests that the isolates represent a closely related species group with similar morphology and life cycles (Vávra and Becnel, 2007).

Life Cycle of Edhazardia aedis

Edhazardia aedis, a pathogen of *Ae. aegypti*, is an example of a microsporidium with a complex life cycle, involving four spore types (Fig. 7.6C), but lacking an intermediate host that is typical for the *Amblyospora* species. It is horizontally transmitted via ingestion of spores as well as vertically transmitted.

Germination of mature lanceolate-shaped spores in the midgut lumen of larvae results in infection of the epithelial cells of the gastric caeca where the microsporidium asexually produces uninucleate, pyriform gametes that have a distinctive double-membraned papilla on the plasmalemma. Plasmogamy produces diplokaryotic stages that develop into small binucleate spores (a form of primary spores) that germinate and disseminate *E. aedis* to other host tissues; the oenocytes in the host hemolymph appear to be a primary target tissue. Most lightly to moderately infected mosquito larvae survive the early infection sequences to develop to the adult stage. In adult mosquitoes, *E. aedis* again multiplies asexually in the oenocytes. The oenocytes move through the hemocoel, coming into proximity with the ovaries in the female mosquitoes. After the female mosquito takes a blood meal, a second binucleate spore, the transovarial spore, is formed and infects the ovaries of the host. This larger, oblong spore infects the filial

generation, but fecundity and longevity are reduced in infected adult females (Becnel *et al.*, 1995), as is success at blood feeding (Koella and Agnew, 1997). *Edhazardia aedis* infects the fat body tissues of the transovarially infected larval host and undergoes a third merogony followed by two types of division. Meiotic division occurs but is abortive, rarely producing meiospores, similar to the meiotic division that produces abortive octospores in the lepidopteran pathogen *Vairimorpha imperfecta* (Canning *et al.*, 2000). A second type of division by nuclear dissociation forms two haploid cells, each of which undergoes sporogony to form large numbers of uninucleate spores. The transovarially infected host dies, releasing infective spores in the aquatic environment that are ingested by susceptible conspecific larvae to complete the cycle. Although infective in the laboratory to a number of mosquito species, *E. aedis* can only complete the full life cycle in *Ae. aegypti* (Becnel and Johnson, 1993; Andreadis, 1994).

7.3.6. Epizootiology and Host Population Effects

Microsporidia infecting insects at the population level are frequently host density dependent (Thomson, 1960; Andreadis, 1984; Streett and Woods, 1993; Briano *et al.*, 1995a). With the exception of laboratory storage, there are no data to suggest that the infective spores survive longer than a year in the environment (Becnel and Andreadis, 1999), so a build-up of long-lived infective units, similar to the resting spores of entomophthoralean fungi (Weseloh and Andreadis, 2002), is unlikely. Enzootic prevalence of microsporidian infection varies depending on specific pathogen–host interactions, from occasional or continuous presence at low levels to widely fluctuating levels that increase greatly when host density is high, the latter suggesting a relatively important role in the population dynamics of the host.

Because immature insects infected with microsporidia often develop slowly, they are potentially vulnerable to biotic and abiotic mortality factors for a longer period than are healthy insects (Maddox *et al.*, 1998). Impacts on the host population caused by delayed development and other sublethal effects that are typical of microsporidian infections, particularly reduced fecundity and, frequently, transovum or transovarially infected offspring, may be evidenced in the generation following an epizootic rather than in the generation experiencing peak prevalence (Maddox *et al.*, 1998; Régnière and Nealis, 2008). This type of host population response has been documented, for example, in spruce budworm infected with *Nosema fumiferanae* (details in Section 7.4.6), the mosquito *Ochlerotatus cantator* infected with *Amblyospora connecticus* (Andreadis, 2005), gypsy moth infected with *Vairimorpha disparis* (Pilarska *et al.*, 1998), and second generation *O. nubilalis* infected with *N. pyrausta* (Hill and Gary, 1979). Some microsporidian species do, however, directly affect the host generation in which prevalence increases occur. Thus, first generation *O. nubilalis* declined as prevalence increased in a two-year study in Connecticut (Andreadis, 1984) and, notably, high density populations of the caddisfly, *Glossosoma nigrior*, appeared to collapse almost completely as microsporidian infection prevalence increased in one season (Kohler and Wiley, 1992).

Seasonal epizootics are common for some insect–microsporidian interactions, including *O. nubilalis* and *N. pyrausta* (Hill and Gary, 1979) and several species of microsporidia and their mosquito hosts (Andreadis, 2007). For populations of other insect species, microsporidian infection may be occasional and rarely epizootic, for example, *N. lymantriae* in gypsy moth populations (Pilarska *et al.*, 1998) and *N. bombi* in populations of *Bombus impatiens* (Cameron *et al.*, 2011). In these latter interactions, microsporidia may never be a major cause of mortality but probably serve as components of the natural enemy complex of their hosts that regulate population densities.

7.3.7. Host Specificity

Microsporidia as a group have been variously reported to be relatively host specific or, primarily based on laboratory host range studies (Sprague *et al.*, 1977b), to have a generally broad host range. Indeed, some microsporidian species do infect multiple insect species. *Nosema bombi*, for example, was reported to infect 22 bumble bee species in the USA (Cordes, 2010) and at least eight bumble bee species in western Europe (Tay *et al.*, 2005), *P. locustae* is known to infect large numbers of grasshopper species (Lange, 2005), and *Cystosporogenes* sp. (probably *Cystosporogenes operophterae*) was recovered from 21 species in eight lepidpoteran families collected in two small research sites in Slovakia (Solter *et al.*, 2010). There are, in addition, records of hymenopteran parasitoids acquiring microsporidian infections from their lepidopteran hosts (Cossentine and Lewis, 1987; Futerman *et al.*, 2006) and of a microsporidian pathogen of mosquitoes infecting humans (Becnel and Andreadis, 1999). Nevertheless, few of the many microsporidian entomopathogens reviewed by Sprague (1977b) have been isolated in field populations of putative host species that were found to be susceptible in laboratory bioassays.

Several laboratory evaluations have shown that infections in non-target hosts are often atypical and suboptimal and, while some non-target insects fed high

dosages of spores may develop patent infections, these infections often are not horizontally and/or vertically transmitted to conspecific susceptible individuals (Andreadis, 1989a, 1994; Solter and Maddox, 1998b; Solter *et al.*, 2005). Some microsporidian species with apparently broad host ranges may actually represent a complex of biotypes, each of which only readily infects a restricted number of hosts. For instance, *N. bombi* infecting bumble bees may represent multiple strains that are each relatively host specific (Schmid-Hempel and Loosli, 1998; Larsson, 2007).

Infections in human hosts by the mosquito pathogen *Anncaliia* (= *Nosema*, = *Brachiola*) *algerae* raised the first concerns about safety of entomopathogenic microsporidia, and *A. algerae* arguably has the broadest known microsporidian host range. Originally isolated from the mosquito *Anopheles stephensi* (Vávra and Undeen, 1970), it has been grown at 37°C in mammalian/human cell cultures (Moura *et al.*, 1999; Lowman *et al.*, 2000), in warm water fish cell culture (Monaghan *et al.*, 2011), and in the extremities of mice (Undeen and Maddox, 1973; Undeen and Alger, 1976). It has also been isolated from corneal epithelial cells (Visvesvara *et al.*, 1999), muscle tissue (Coyle *et al.*, 2004), and vocal cords (Cali *et al.*, 2010) of human patients. Although reported human cases are extremely rare, *A. algerae* is considered to be an emergent disease organism, particularly of concern for humans who are immunocompromised (Cali *et al.*, 2005). Two other entomopathogenic microsporidia are of some concern, although no vertebrate infections have been recorded thus far. *Vavraia culicis* is genetically closely related to *Trachaepleistophora homminis*, a species described from human patients but capable of infecting mosquitoes (Weidner *et al.*, 1999), and *E. romaleae*, isolated from lubber grasshoppers, is the only known species in the genus *Encephalitozoon* that is not a vertebrate pathogen (Lange *et al.*, 2009).

Despite these concerns, with the exception of *A. algerae*, there is no evidence that other entomopathogenic microsporidia are pathogenic to vertebrate animals. The safety issues that are important to consider for their use in biological control programs primarily involve the potential susceptibility of non-target native insects to introduced pathogens (Solter and Becnel, 2003). Because microsporidian species vary widely in breadth of host range, both field and laboratory studies are needed to evaluate the potential for a species introduced as a classical biological control agent to "jump" to a non-target host. The physiological (laboratory) host range is important to consider should microsporidia be used as microbial insecticides because inundative field rates may be similar to the typically high dosages used in laboratory bioassays. Microsporidia that are relatively host specific and are inoculatively released in augmentative or classical biological programs are far less likely to affect non-target species, particularly if release does not involve inundative methods such as spraying (Solter *et al.*, 2010). Host specificity studies were conducted for several of the microsporidia covered in the following case histories and are mentioned in the context of the various biological control programs.

7.4. BIOLOGICAL CONTROL PROGRAMS: CASE HISTORIES

This section presents several long-term studies of microsporidia as potential biological control agents. Although not every attempt has been successful, the information gathered is a resource for increased understanding of microsporidian–host interactions and provides a framework for future considerations of the role of microsporidia in biological control programs.

7.4.1. Use of Microsporidia in Biological Control Programs

Microsporidia are best suited for use as natural controls, introduced as classical biological control agents, and in augmentative and conservation biological control programs (Lacey *et al.*, 2001). They have not been found to be suitable as inundatively released microbial insecticides for two primary reasons. First, even at relatively high application rates, infections are typically chronic rather than acute, with the major effects on the host being reduced lifespan and fecundity. While these effects have been documented to suppress populations of insect pests, microsporidia are not sufficiently fast acting, nor do they typically produce the high mortality rates expected for chemical or microbial insecticides (Lacey *et al.*, 2001; Solter and Becnel, 2007). Second, microsporidia cannot be inexpensively cultured in artificial media or fermentation tanks. They require living cells to reproduce, therefore, *in vivo* production requiring mass rearing of hosts or cell culture is needed for mass production. Both methods are costly and labor intensive. Most species of microsporidia are difficult to culture successfully or produce fewer spores in cell culture than in the host (Khurad *et al.*, 1991; Kurtti *et al.*, 1994). Despite 50 years of research on microsporidia of insect pests, only one species, *P. locustae*, a pathogen of a suite of grasshopper species, has been registered by the US Environmental Protection Agency (USEPA) for use as a microbial insecticide.

There are, nevertheless, numerous situations where adequate control of established pests can be achieved by manipulating their naturally occurring pathogens.

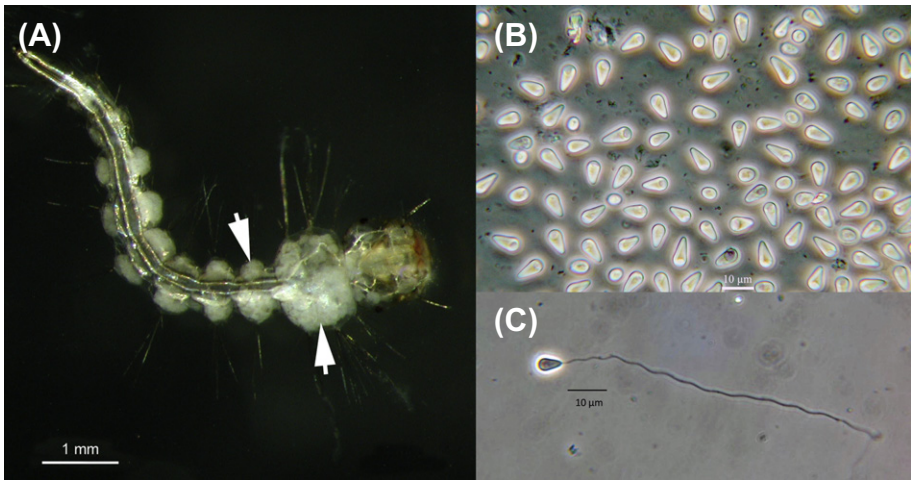


FIGURE 7.7 (A) *Edhazardia aedes* infecting fat body tissues (arrows) of mosquito host; (B) *E. aedes* environmental spores; and (C) germinating spore with everted polar filament. *(Photos by J. J. Becnel.)*

Outbreaks of native pests in economically important areas, and accidental or intentional introduction of non-native plants and animals, have created serious global issues concerning habitat damage and economic loss. The development of methods to suppress outbreak and invasive species while avoiding collateral damage to populations of native species is critical for many systems. Microsporidia are present in insects as naturally occurring primary pathogens. Although they do not function well as microbial insecticides, their more subtle effects can have important impacts on host population dynamics and, in the context of the natural enemy complex of a pest species, they may be important regulators of outbreak species (Anderson and May, 1980).

This section includes descriptions of several long-term research efforts to evaluate the role and potential of microsporidia as biological control agents for several major pests in a variety of managed and natural habitats. These laboratory and field studies include general biology and taxonomy of the pathogens, efficacy testing, host density dependence and persistence in host populations, and evaluation of host specificity.

7.4.2. Aquatic Diptera

Microsporidia are generally the most commonly found pathogens in natural populations of aquatic Diptera but usually occur at very low prevalence levels. The Culicidae have been the focus of much of the research on microsporidia in aquatic Diptera because of their medical importance and microbial control potential. Microsporidia are also common in the Simuliidae, Chironomidae, and Ceratopogonidae.

Microsporidia were seriously considered as biological control agents for mosquitoes because of their ability to cause larval epizootics, continuously cycle within a host population, and spread to new habitats. The idea of utilizing these natural enemies of mosquitoes as manipulative control agents was perhaps first raised by Kudo (1921), who suggested that larval sites might be contaminated with microsporidian-infected mosquito tissues. Unaware at that time of the mechanism of transovarial transmission, he further suggested that infected adults could distribute the parasite to new sites that "may escape our watchful eye" by dying during oviposition.

The first microsporidia evaluated for mosquito control were those with relatively simple life cycles that were directly infectious to the host. The initial attempt to introduce a microsporidian parasite was presumably with *V. culicis* (Fig. 7.7) against *Cx. pipiens fatigans* on the South Pacific island of Nauru (Reynolds, 1972). The second attempt was with *A. algerae* against *Anopheles albimanus* in Panama (Anthony *et al.*, 1978). Both of these studies demonstrated that microsporidian parasites could be introduced into natural larval sites and result in considerable infection that reduced adult longevity and fecundity; they did not, however, cause significant larval mortality or persist and spread at levels considered important for population reduction. Because of public health and safety concerns (see Section 7.3.7 and reviews by Becnel *et al.*, 2005, and Andreadis, 2007), *A. algerae* and *V. culicis* can no longer be considered as viable biological control agents.

Two discoveries led to a renewed interest in microsporidia as microbial control agents of mosquitoes during the 1980s and 1990s. The first discovery was the involvement of an intermediate copepod host in the life cycle of *Amblyospora* (Sweeney *et al.*, 1985) and *Parathelohania* (Avery, 1989; Avery and Undeen, 1990), documenting for the first time the mechanism for horizontal transmission in these genera. The second was the identification of a new genus of microsporidia, *Edhazardia*, that is both

horizontally and vertically transmitted but does not require an intermediate host (Hembree, 1979; Becnel *et al.*, 1989).

Much of the information concerning the life cycles and evaluations of polymorphic microsporidia has been derived from studies on *Amblyospora dyxenoides* (Sweeney *et al.*, 1988; Sweeney and Becnel, 1991), *A. connecticus* (Andreadis, 1988a), and *E. aedis* (Becnel *et al.*, 1989), but little recent work has been conducted. This discussion will focus on *E. aedis*, a species that does not require an intermediate host, and *A. connecticus*, an example of a species that requires a copepod intermediate host.

Edhazardia aedis

Edhazardia aedis, a pathogen of *Ae. aegypti*, was isolated in Thailand (Hembree, 1979). This pathogen has a complex life cycle involving both horizontal and vertical transmission affecting two successive generations of the host (Hembree, 1982; Hembree and Ryan, 1982). Usually, one sporulation sequence occurs in the adult female (infected orally as a larva) and results in the formation of binucleate spores. These spores are involved in vertical transmission of *E. aedis* to the subsequent generation via infected eggs. In infected progeny, there are two sporulation sequences in larval fat body but, owing to the abortion of one of the sequences, only one viable spore type is produced (Becnel *et al.*, 1989). Larval death results in the release of uninucleate spores that are responsible for horizontal transmission when ingested by larvae. This developmental sequence leads to the formation of binucleate spores in the adult to complete the cycle.

There are two known deviations from this parental host−filial host alternation that may play important roles in maintenance of *E. aedis* under natural circumstances (Becnel *et al.*, 1989). In these instances, the parasite completes its development through repeated cycles of horizontal transmission or, alternatively, by repeated cycles of vertical transmission.

Edhazardia aedis was transmitted to its natural host, *Ae. aegypti*, and to eight alternate hosts in the laboratory: *Ae. albopictus*, *Ae. triseriatus*, *Ae. taeniorhynchus*, *Ae. atropalpus*, *Ae. vexans*, *Anopheles quadrimaculatus*, *Orthopodomyia signifera*, and *Toxorhynchites rutilus rutilus* (Becnel and Johnson, 1993; Andreadis, 1994). Thirteen other mosquito species were not susceptible to *E. aedis*, including all species of *Culex* tested. In all susceptible hosts, the microsporidium underwent normal development but transovarial transmission was successful only in *Ae. aegypti*. Therefore, while a variety of mosquito species representing diverse genera is physiologically susceptible to *E. aedis per os*, the pathogen is specific for *Ae. aegypti*. Common non-target aquatic organisms were not susceptible to infection by *E. aedis* and, hence, there was no mortality due to *E. aedis* (Becnel, 1992b).

Edhazardia aedis causes larval death as a result of the highly efficient mechanism of vertical transmission, with approximately 95% of the progeny infected (Hembree, 1982; Hembree and Ryan, 1982; Becnel *et al.*, 1995). Spores released from these infected progeny are infectious to all instars of *Ae. aegypti* and result in infected adults. The influence of the microsporidium *E. aedis* on the survival and reproduction of its mosquito host *Ae. aegypti* was studied in the laboratory (Becnel *et al.*, 1995). Survival, fecundity, egg hatch, and percentage emergence for four gonotrophic cycles were compared for control and infected mosquitoes. Infected females oviposited 70% fewer eggs and percentage hatch was lower than for control females. Emergence in progeny of infected female *Ae. aegypti* was significantly less than for control mosquitoes in all gonotrophic cycles. The reproductive capacity (R_o) for control and infected adults was 168 and four, respectively, representing a 98% decrease. A semi-field study that involved the inoculative and inundative release of *E. aedis* produced encouraging results (Becnel and Johnson, 2000). Limitations to incorporating *E. aedis* into an integrated control program are the high costs involved in production in host mosquitoes and methods to store the fragile spore stage. The only possible field application of this microsporidian parasite would be as part of a classical biological control program to establish *E. aedis* in naïve host populations for long-term control (Becnel, 1990).

Ecological and epizootiological studies of *E. aedis* in natural populations of *Ae. aegypti* have not been completed. Optimism regarding the role of *E. aedis* as part of a program to control *Ae. aegypti* is based on a number of desirable traits determined in laboratory and semi-field studies. These studies have demonstrated that both the vertical and horizontal components of the life cycle of *E. aedis* are highly efficient in providing the means for the parasite to become established, persist, and spread in populations of *Ae. aegypti*. The profound effect on reproductive capacity of infected adults suggest the *E. aedis* can have a strong influence on host population dynamics (Becnel *et al.*, 1995). Both inoculative and inundative release strategies were evaluated using *E. aedis* against a semi-natural population of *Ae. aegypti* (Becnel and Johnson, 2000). Inoculative release resulted in dispersal of *E. aedis* to all containers within the study site over a 20-week period. Inundative release eliminated the population of *Ae. aegypti* within 11 weeks of introduction. Good persistence is expected in release sites owing to life cycle flexibility with dissemination to other mosquito-inhabiting sites by means of vertical transmission. Survival during dry periods occurs within the mosquito eggs, where the pathogen can survive for the life of the egg (Becnel *et al.*, 1989). In addition, an obligatory intermediate host is not required for horizontal transmission.

These desirable traits of *E. aedis*, including host specificity, support the belief that this pathogen can play an important role as a classical biocontrol agent in developing strategies to control *Ae. aegypti*. Ideally, *E. aedis* would be introduced via inoculative releases (rather than inundative releases), lessening the need for mass production. For this method to be successful, the pathogen must become permanently established or augmented seasonally to maintain acceptable levels of control.

Amblyospora connecticus

Amblyospora connecticus is a pathogen of the brown saltmarsh mosquito, *Aedes cantator*, from Connecticut, USA. A characteristic of most known species of *Amblyospora* is the requirement of two generations of the mosquito host and an obligate sequence in a copepod intermediate host to complete the life cycle (Andreadis, 1988a, 1990b).

Like *E. aedis*, the life cycle of *A. connecticus* involves both horizontal and vertical transmission in the mosquito host. In the infected adults, binucleate spores formed after a blood meal are responsible for transovarial transmission to progeny. Some female and most male progeny from infected adults develop fatal fat body infections that produce a distinct spore type, the meiospores. Released meiospores are infectious *per os* to the copepod intermediate host, *Acanthocyclops vernalis*. A sporulation sequence in the intermediate host ends with the production of uninucleate spores that are infectious *per os* to a new generation of the mosquito host. In larval mosquitoes, a developmental sequence leads to the formation of binucleate spores in adults to complete the cycle.

Amblyospora connecticus was experimentally transmitted to four alternate mosquito hosts; *Ae. atropalpus*, *Ae. epacticus*, *Ae. sierrensis*, and *Ae. triseriatus* (Andreadis, 1989a). Binucleate spores were formed only in *Ae. epacticus* and none of the alternate hosts was able to transmit the parasite vertically to progeny. Therefore, *A. connecticus* was unable to complete its life cycle in any of the alternate hosts, demonstrating a high degree of specificity for *Ae. cantator*. Fifteen other mosquito species belonging to the genera *Aedes*, *Anopheles*, *Culex*, *Culiseta*, and *Psorophora* were not susceptible.

Seasonal epizootics of *A. connecticus* in *Ae. cantator* larvae occur each fall (autumn; October–November) with infection rates of up to 100%, and some sites in Connecticut had an overall prevalence of 98% (Andreadis, 1983). These lethal infections are a result of the synchronous hatch of transovarially infected eggs and serve to produce meiospores that infect the intermediate host, *A. vernalis*. A small sample of field-collected infected *Ae. cantator* were examined to determine whether infection with *A. connecticus* is detrimental to reproductive success. Of 195 females examined, four were found to be infected with *A. connecticus*. There was no significant difference in fecundity or the percentage of eggs that hatched between healthy and infected females (Andreadis, 1983). It was concluded that *A. connecticus* does not detrimentally affect the reproductive success of *Ae. cantator*.

Life-cycle Based Management Strategies

Knowledge about the dynamics of a microsporidia–mosquito system can be utilized as part of a mosquito management project and is demonstrated using the extensive ecological and epizootiological data collected for *A. connecticus* (Andreadis, 1988b, 1990a). The mosquito host, *Ae. cantator*, is a typical multivoltine, saltmarsh species. It overwinters in the egg stage and may produce up to four generations a year in the north-eastern USA. Eggs hatch in spring (March) and the first adults emerge by May. Subsequent generations appear periodically as the saltmarsh pools are flooded by rain and/or high tides. The copepod intermediate host, *A. vernalis*, is a common species that has one to two generations a year in the north-eastern USA and overwinters as a diapausing fourth or fifth stage copepodid. It is abundant during the spring and fall but aestivates during the summer months.

In a three-year study on natural ecology, it was demonstrated that there is a well-defined seasonality to the transmission cycles and epizootics involving *A. connecticus* (Andreadis, 1988b). Larval epizootics as a result of transovarial transmission occur each fall and routinely produce infection levels of 80–100%. These epizootics in *Ae. cantator* result in larval death and release of meiospores which coincides with the fall appearance of the intermediate host, *A. vernalis*. Copepods become infected by ingesting meiospores but development of *A. connecticus* is arrested until the following spring when winter dormancy ends. Sporulation of *A. connecticus* and subsequent death of the copepod coincides with the hatch of *Ae. cantator* in the spring when horizontal transmission to the mosquito host occurs. Infected female adult mosquitoes produced at this time lay infected eggs throughout the summer. Infected eggs hatch synchronously in the fall, causing epizootics, but few hatch during the summer, even if flooded.

The two major events identified as critical to the maintenance of *A. connecticus* are (1) the fall epizootic responsible for significant larval mortality of *Ae. cantator* and, concurrently, the infection of the copepod intermediate host to provide for overwintering of the parasite; and (2) the spring epizootic, when spores formed in the copepod are responsible for horizontal transmission to larval *Ae. cantator* and result in infected adults. Awareness of these critical windows in the maintenance of *A. connecticus* suggests a control strategy that precludes larviciding these habitats during the spring and fall as this would be counterproductive and disrupt the natural balance of the disease cycle. Larviciding these habitats during the summer is suggested by life

cycle data that demonstrate an absence of infected mosquitoes as well as copepods during the summer months. This strategy would also target the healthy part of the mosquito population that had escaped infection by *A. connecticus*. In addition, *A. connecticus* was successfully introduced and maintained in a field population of *Ae. cantator* via infected *A. vernalis* (Andreadis, 1989b). This demonstrates that augmentation can be used as part of a classical biological control project to introduce microsporidia with complex life cycles involving an intermediate host.

Microsporidia for mosquito management has been proposed as one component of the natural complex of regulatory factors utilized for control. This approach recognizes that eradication of the target mosquito is an unrealistic goal but with a combination of physical, cultural, chemical, and biological control methods, mosquito vectors and pests can be regulated.

7.4.3. Lepidopteran Pests in Row Crop Systems

Classical or conservation biological control of plant pests should be appropriate for row crop systems, including those ranging from small truck farms to large-scale grain acreage. Most arthropod herbivore populations are, indeed, limited to a certain extent by their complex of natural enemies, even in crop monoculture, and many seed and fruit crops can tolerate some pest damage to foliage without damage to the product. Stem damage causes weakness and reduced yield (Bode and Calvin, 1990), however, and few markets will bear significant damage to fruit or other edible plant parts. The economics involved with pest control in these high value and ecologically artificial systems demand fast, inexpensive, and nearly complete control at a level that most natural enemies cannot achieve. Nevertheless, natural enemies, including microsporidia, can be factored into treatment decisions that benefit the producer, consumer, and environment. Although microsporidia have been isolated from a number of field crop pests, only two species, *Nosema pyrausta* in *Ostrinia nubilalis*, and *Vairimorpha necatrix* isolated from several noctuid species, have been extensively studied to evaluate host–pathogen interactions and effects on host population dynamics. *Vairimorpha necatrix* is a virulent and promising pathogen that can produce mortality in the range of a nucleopolyhedrovirus and may have some potential for use in greenhouse production (Down *et al.*, 2004). Unfortunately, *V. necatrix* has been disappointing in field trials because it is not persistent at levels that are effective for the control of targeted noctuid pests (Brooks, 1980). In contrast, *N. pyrausta* is particularly important as a persistent and primary pathogen of *O. nubilalis*.

Although *O. nubilalis* larvae have a wide host range that includes a variety of weeds, grasses, and vegetable crops with stems or fruit sufficiently large enough to support boring, it has, since its introduction to the USA in the early 1900s, become the most serious pest of corn in North America. The life cycle includes one to three generations annually; in the Midwest, there are typically two. The larvae first feed on leaves of the corn host, causing minor damage, then move to leaf whorls and tassels before boring into the stalk to complete their development. Damage includes broken stalks (lodging) and ear shanks, damaged ears (particularly in popcorn and sweet corn), and decreased yield. Losses in the late 1990s were reported to exceed $1 billion/year in the USA alone (CSREES, 2008). Treatments for *O. nubilalis* have included introduced parasitoids and all permitted pesticides from arsenicals and multiple synthetic compounds to microbial insecticides and, since the late 1990s, transgenic corn hybrids expressing *Bacillus thuringiensis kurstaki* (Btk) Cry proteins. *Ostrinia nubilalis* is host to a small number of pathogens, with *Beauveria bassiana* and *N. pyrausta* having the most important effects on population dynamics of the host. Nearly 80 years of research on *N. pyrausta* were recently reviewed by Lewis *et al.* (2009), and the reader is directed to this history for details. Here, the importance of this microsporidian pathogen in *O. nubilalis* population dynamics is briefly described for the two-generation cycle.

Nosema pyrausta is a sister species to the "true" *Nosema* group (Vossbrinck and Debrunner-Vossbrinck, 2005), closely related to *N. bombycis* and, like other members of this microsporidian clade infecting Lepidoptera, infections are chronic and systemic. *Nosema pyrausta* is transmitted both horizontally and vertically, bridging the two (or three) annual generations of the host. Infected overwintering fifth instar larvae have lower survival rates than uninfected larvae (Siegel *et al.*, 1986b), but those that survive are generally able to eclose, mate, and oviposit. Larvae that are transovarially infected contaminate the leaf whorls and newly bored tunnels with spore-laden frass and decomposing infected hosts. Mortality is high in first generation transovarially infected larvae and, thus, prevalence of the disease often declines during the first generation (Siegel *et al.*, 1986b), but the second generation is infected by both disseminated spores and transovarial transmission. This generation enters fifth instar diapause, serving as inoculum for the following spring generation.

Nosema pyrausta prevalence in *O. nubilalis* populations is density dependent (Hill and Gary, 1979; Andreadis, 1986), and enzootic levels are high relative to many other microsporidia–host interactions. Before the use of transgenic corn, it was not unusual to find populations with prevalence levels that fluctuated between 5% and 60% or higher (Hill and Gary, 1979; Andreadis, 1986; Lewis *et al.*, 2006). The well-documented effects of *N. pyrausta* infections on the *O. nubilalis* host are typical for chronic microsporidian infections. In addition to high mortality

rates in overwintering larvae and transovarially infected larvae, adult fecundity is generally reduced (reviewed by Lewis *et al.*, 2009), larval growth rates and development time are slowed (Lewis *et al.*, 1983; Solter *et al.*, 1990), and flight behavior is negatively affected (Dorhout *et al.*, 2011). The effects of *N. pyrausta* are exacerbated (additive effects) by exposure to chemical insecticides (reviewed by Lewis *et al.*, 2009) and Btk (Pierce *et al.*, 2001; Lopez *et al.*, 2010). Evaluations of *O. nubilalis* populations 10 years after the first and, currently, expanded use of transgenic corn hybrids have determined that the use of Bt hybrid corn has not eliminated *N. pyrausta* in *O. nubilalis* populations and it is recommended that care should be taken in the management of Bt corn to conserve this pathogen in *O. nubilalis* integrated pest management (IPM) programs (Lopez *et al.*, 2010).

7.4.4. Grasshoppers and *Paranosema locustae*

The extensive western grasslands of the USA are a region grazed by livestock and plagued by irregular outbreaks of grasshoppers. The pest grasshoppers fall within four main subfamilies in the family Acrididae, with about 10–15 species being major pests (Capinera and Sechrist, 1982; Pfadt, 2002). A diversity of developmental times, foraging preferences, aggregation and migration patterns, and other biological and behavioral traits is represented by the numerous species. Integrating these variable traits with habitat and abiotic factors such as moisture and temperature results in population outbreaks with highly variable and unpredictable intensity and frequency (Lockwood and Lockwood, 2008). In addition, this system is unique in that the spatial scale of the outbreak can be vast, encompassing thousands or even millions of hectares. Forage devastation can occur quickly in localized but dispersed foci (Lockwood *et al.*, 2001). Treatments are most efficacious when applied within a limited time-frame that targets third instar nymphs to suppress grasshopper populations before substantial forage is consumed (Hewitt and Onsager, 1983). Similarly, grasshoppers plague other rangeland ecosystems of the world including northern China (Shi *et al.*, 2009) and the Pampas in Argentina (Lange and Cigliano, 2005).

Given the significant destruction of crops and rangeland forage by grasshopper outbreaks (Hewitt and Onsager, 1983; Joern and Gaines, 1990; Lockwood *et al.*, 2002), grasshoppers have long been the target of intense control programs utilizing various insecticide sprays and baits (Latchininsky and VanDyke, 2006). Insecticide sprays and baits can quickly kill grasshoppers (Quinn *et al.*, 1989), with the latter having less detrimental effects on non-target organisms (Quinn *et al.*, 1990; Peach *et al.*, 1994; McEwen *et al.*, 2000; Foster *et al.*, 2001). With applications of either formulation, the usual objective is to immediately quell the destructive feeding by grasshoppers in that season. It is within this paradigm that the entomopathogen *Paranosema locustae* became the first and, currently, the only microsporidium to be commercially produced and registered for grasshopper control in rangelands (USEPA, 1992; Lockwood *et al.*, 1999).

Paranosema locustae was originally described in 1953, from colonies of African migratory locusts, *Locusta migratoria mirgratoroides*, being reared in England. It was initially named *Nosema locustae* (Canning, 1953), but it has since been placed in a new genus *Paranosema* (Sokolova *et al.*, 2003). Subsequently, the microsporidium was transferred to the genus *Antonospora* (Slamovits *et al.*, 2004) but the change was refuted by Sokolova *et al.* (2005a). In addition to *L. migratoria*, *P. locustae* was identified from several species of North American grasshoppers (Canning, 1962a) and is now known to have an unusually broad host range for a microsporidium, infecting 121 species of Orthoptera (Lange, 2005). The mature infective spores are ellipsoidal in shape, 3.5–5.5 μm in length by 1.5–3.5 μm in width, with a thick spore wall (Canning, 1953, 1962a, b).

Paranosema locustae primarily infects the adipocytes of fat body tissue, resulting in the disruption of the host's metabolism and energy storage. Other tissues can also be infected in heavily infected hosts. Similar to other microsporidian infections, *P. locustae* causes chronic debilitation, which is associated with reduced feeding, development, and fecundity, in addition to increased mortality rates (Canning, 1962b; Henry and Oma, 1974; Ewen and Mukerji, 1980; Johnson and Pavlikova, 1986). Sublethal infections in locusts were associated with a shift from the gregarious form to the less damaging solitary phase (Fu *et al.*, 2010). In extensive infections, the fat body is greatly hypertrophied with spores and acquires an opaque, cream coloration that can progress to a pink and eventually a dull red color.

Horizontal transmission of *P. locustae* is very efficient. Ingestion of vegetation inoculated or contaminated with spores, and cannibalism and necrophagy of infected hosts serve as routes of transmission (Canning, 1962b; Henry and Oma, 1981; Ewen and Mukerji, 1980). The third instar nymph is the stage most susceptible to acquiring infection. Infection at this stage development causes the highest initial mortality within a population; yet, a high prevalence of infection is maintained in survivors (Canning, 1962b; Henry *et al.*, 1973). In addition, *P. locustae* can be transmitted transovarially, with infected nymphs potentially serving as inoculum for horizontal transmission via cannibalism (Raina *et al.*, 1995). Observations of vertical transmission in hatchlings from field-collected eggs, however, revealed low levels of infection that were considered inadequate to establish infections in the following year (Ewen and Mukerji, 1980).

Early assessments considered the virulence of *P. locustae* to be insufficient to protect crops despite high nymphal mortality and efficient transmission (Canning, 1962b). However, initial field studies with *P. locustae* as an augmentative biological control were encouraging (Lockwood *et al.*, 1999). Locust populations were inoculated by applying spores to wheat bran that grasshoppers ingested, resulting in a peak infection prevalence of 43% and population reductions of some species. There was also evidence of reduced fecundity in infected females (Henry, 1971a). The efficient horizontal transmission and moderate virulence in adult hosts enabled large quantities of spores to be produced *in vivo*. This afforded the opportunity to further develop and evaluate *P. locustae* bait (Henry *et al.*, 1973; Ewen and Mukerji, 1980; Henry and Oma, 1981). The broad host range of *P. locustae* was considered by some to be a desirable attribute, as many species of grasshoppers damage crops and different developmental phenologies among species provide a continuous availability of susceptible hosts to maintain infections (Henry, 1971b), but it has also been noted that the variable susceptibilities among species would contribute to inconsistent efficacy (Lockwood *et al.*, 1999). Another advantage is that spores can be stored by freezing, but while storage capability made conducting trials with *P. locustae* logistically easier, freezing was reported to be a hindrance (Henry and Oma, 1974; Lockwood *et al.*, 1999). *Paranosema locustae* was registered as *Nosema locustae* (this species name is still recorded on EPA labels) and produced commercially in the USA beginning in the 1980s by a variety of companies and marketed under trade names such as Nolo Bait™, Semaspore™, and Grasshopper Attack™ (Lockwood *et al.*, 1999). Nolo Bait is still produced by a company in Colorado, and Semaspore is produced by a company in Montana. The USEPA (1992) produces a factsheet with further details on *P. locustae* bait products. The efficacy of *P. locustae* bait has been variable in numerous field studies with typical population reductions of 30% accompanied by 20–40% infection prevalence among survivors (Johnson, 1997). This level of control, however, would be perceived inadequate by end-users when compared to 70–95% control with insecticides (Vaughn *et al.*, 1991).

The construal of *P. locustae* as a microbial insecticide brought inappropriate and unfulfilled expectations of fast and thorough suppression of grasshopper populations and damage. This shrouded the original concept of using *P. locustae* as an augmentative biological control agent to improve long-term suppression of grasshoppers (Henry and Oma, 1981; Lange and Cigliano, 2005). Attempts to increase infection prevalence and population reductions of grasshoppers with inundative applications of *N. locustae* have not been successful. For example, two successive annual applications of the commercially produced *P. locustae* bait (Nolo Bait) did not sufficiently increase prevalence among or the severity of infection in infected grasshoppers or result in significant population impacts, nor were sublethal effects of reduced fecundity and feeding apparent. These results were for both years of the applications and the subsequent third season and resulted in the conclusion that crop protection with *P. locustae* alone would be difficult to detect (Johnson and Dolinski, 1997). Infections of some grasshopper species reached 35%, however, which suggested a value for including *P. locustae* as a biological control component of an IPM program or in areas that can tolerate some levels of damage (Johnson and Dolinski, 1997; Vaughn *et al.*, 1991). The inconsistent efficacy of *P. locustae* bait relative to chemical insecticides, the high cost of *in vivo* spore production, and limited long-term spore storage have made marketing commercial formulations difficult in the USA and internationally. Even extensive government-subsidized applications of *P. locustae* in China were perceived to be inadequate by growers, with grasshopper reductions of 60% or less and slow mortality times (Lockwood *et al.*, 1999).

Pathogen enzootics and epizootics are generally thought to be among the many factors that influence grasshopper populations. The degree to which pathogens play a role in diminishing the frequency and severity of grasshopper outbreaks is difficult to ascertain (Joern and Gaines, 1990). *Paranosema locustae* was introduced into naïve grasshopper populations in several locations in Argentina in 1978–1982 using inocula from North America. Formal monitoring of the fate or impact of the introductions was not reported until the pathogen was detected in 1991 in the western Pampas region. *Paranosema locustae* continued to be observed over a span of 11 consecutive years. The sustained infections suggested efficient horizontal transmission and even a possible persistent decline in population abundance and frequency of grasshopper outbreaks (Lange and Cigliano, 2005). Similar conclusions were reported from China where a single application of *P. locustae* bait resulted in variable population declines and 10-year persistence. Indeed, *P. locustae* has been applied extensively in China for over 20 years and is thought to be useful for grasshopper and locust management when populations are moderate (Shi *et al.*, 2009). In both Argentina and China, the use of *P. locustae* is an example of "neoclassical" biological control, where an exotic organism is introduced to control a native pest (Lockwood, 1993). The establishment of an exotic biocontrol agent elicits concern regarding its impact on non-target organisms, shifts in species assemblages, and other components of the rangeland. While shifts in grasshopper species have been reported in Argentina, they are considered to be part of the inevitable changes to the Pampas as agroecosystems become prevalent (Lange and Cigliano, 2005). With the exception of China, the application of *P. locustae* bait for grasshopper suppression has dwindled. However,

P. locustae bait was reregistered by the USEPA and is sold in the USA mainly as an organic treatment for grasshoppers.

7.4.5. Fire Ants in Urban Landscapes

Fire ants are invasive stinging insects native to South America that were inadvertently introduced into the USA in the early 1930s. They currently infest the southern USA and parts of California and, since 2000, infestations have established in Australia, China, and Mexico (Ascunce *et al.*, 2011; Sanchez-Peña *et al.*, 2009). Fire ants have become ubiquitous within urban, agricultural, and natural landscapes. They are most noted for the painful, burning stings they inflict on humans, pets, and livestock. A conservative estimate is that 1% of people stung in the USA are potentially allergic to the venom and are at risk for anaphylaxis (Triplett, 1976). In addition, fire ants can damage crops, and the dominance of these ants in natural landscapes has reduced biodiversity (Wojcik *et al.*, 2001).

Despite the availability of insecticides that can effectively suppress fire ant populations, it is often logistically impractical or cost prohibitive to treat many infested sites. Untreated or unmanaged infested areas are key sources of reinfestations in areas cleared of fire ants or are the source of new infestations. Using entomopathogens as biological control agents is considered to be one of the few viable methods of sustainable control for invasive ants.

Kneallhazia solenopsae

Extensive efforts have been made to utilize the microsporidium *Kneallhazia solenopsae* for the biological control of fire ants. This pathogen was first reported from *Solenopsis invicta* in 1974 and described in 1977 as *Thelohania solenopsae*, but was subsequently placed in a new genus, *Kneallhazia* (Allen and Buren, 1974; Knell *et al.*, 1977; Sokolova and Fuxa, 2008).

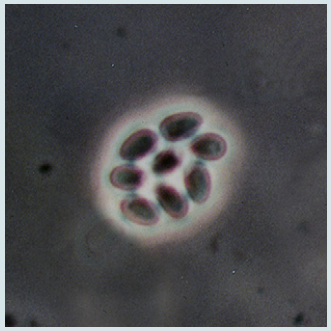
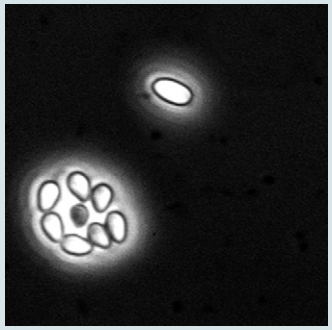
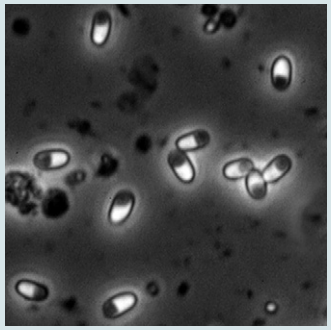
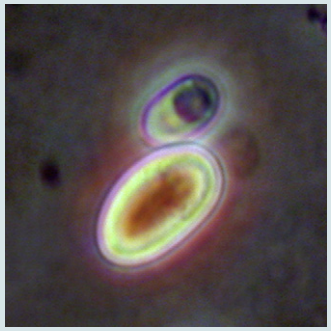
Four spore types have been reported from *K. solenopsae* (Table 7.2). These are as follows: (1) Octets of pyriform, uninucleate, meiospores within sporophorous vesicles (octospores) are the most abundant and are typically found in the worker and reproductive caste of the adult ants. They are concentrated, though not exclusively, in the fat body tissues. (2) Diplokaryotic, *Nosema*-like, free spores (not enclosed within a vesicle) are also found in adult ants of both castes, and occasionally in larvae and pupae. These spores are ovoid and slightly larger than individual meiospores and are much less abundant. (3) Exclusively found in fourth instars and pupae, the diplokaryotic primary spores are ovoid with a distinctive posterior vacuole. (4) Megaspores are elongated ovals and the largest of the spores. They are diplokaryotic and, while most frequently observed in the adult reproductive caste, they are also present in adult worker ants, fourth instar larvae, and pupae.

Although the functions of the various spores of *K. solenopsae* are not definitively known, the biology and a hypothetical life cycle have been developed (Sokolova and Fuxa, 2008). In general, latent infections are found in the early immature stages (eggs, first to third instars) of fire ants. Mature spores were not observed by light microscopy in these stages, but infections were detected by polymerase chain reaction (Briano *et al.*, 1996; Valles *et al.*, 2002; Sokolova and Fuxa, 2008). It was proposed that infections in the early immature stages precede the proliferation of primary spores in fourth instars and pupae. Primary spores initiate the rapid distribution of infection within the host and serve as the initial source of the *Nosema*-like spores and megaspores. *Nosema*-like spores are thought to be involved in the early stage of pathogenesis, autoinfecting adipocytes. This infection sequence switches to megaspore and intensive meiospore production, which results in a conglomerate of spores enclosed within the expanded membrane of the now dysfunctional adipocyte, forming cysts, or sporocytosacs (Sokolova *et al.*, 2005b) (Fig. 7.8). While meiospores are by far the most copious spore type, their function is not understood. Megaspores are regularly observed in the cells of muscle, tracheoles, and fat bodies associated with ovaries and testes of fire ant reproductives. Germinated megaspores have also been observed in the ovaries of inseminated queens (Sokolova and Fuxa, 2008). Coupling these observations with the detection of vegetative stages in eggs (Briano *et al.*, 1996) provides evidence for megaspore involvement in the transovarial transmission of *K. solenopsae*.

Infections of *K. solenopsae* have been initiated in fire ant colonies by introducing live, infected fourth instar larvae and pupae (Williams *et al.*, 1999; Oi *et al.*, 2001); however, the mechanism of horizontal transmission between individual ants is not known. *Per os* inoculation of suspensions of the primary, *Nosema*-like, and meiospore types have not resulted in infections (Shapiro *et al.*, 2003). Adult *S. invicta* workers and virgin female reproductives possess an efficient filtering system that traps and removes solid particles from ingested liquids. They are able to filter particles 0.5−0.75 μm or larger in diameter (Glancey *et al.*, 1981; Petti, 1998; Oi, unpubl. data). Thus, *K. solenopsae* spores theoretically should not be ingested by adult *S. invicta* since all known spore types are considerably larger than 0.75 μm (Table 7.2). However, fourth instar larvae have ingested maximum particle sizes of 45.8 μm (Glancey *et al.*, 1981). While larvae are capable of ingesting spores and become infected, how adult queens become infected is yet to be determined (Chen *et al.*, 2004; Sokolova and Fuxa, 2008).

The ability to initiate colony infections by introducing live, *K. solenopsae*-infected brood has facilitated the evaluation of this pathogen as a biological control agent. Laboratory inoculations have resulted in brood reductions

TABLE 7.2 ***Kneallhazia solenopsae*** **Spore Types and Size, Primary Fire Ant Stages and Castes, and Spore Images and Descriptions**

Spore Type	Size (μm)	Primary Fire Ant Stages (Occasional Host)	Spore Image	Spore Description
Uninucleate meiospore (octospore)	3.3 × 1.95[a]	Adult workers, queens, alates (pupae)[b c]		Eight meiospores in sporophorous vesicle
Binucleate free or *Nosema*-like spore	4.9 × 1.85[a]	Adult workers, female reproductives (larvae, pupae)[c]		Free spore (right) and octet of meiospores
Diplokaryotic primary spore	4.5 × 2.3[d]	Pupae (larvae)[e]		Primary spores, with large posterior vacuole
Binucleate megaspore	6.2 × 3.6[c]	Queens, alate females, workers, larvae, pupae[e]		Primary spore and larger megaspore

[a] *Knell* et al. *(1977);*
[b] *Briano* et al. *(1996);*
[c] *Sokolova* et al. *(2004);*
[d] *Shapiro* et al. *(2003);*
[e] *Sokolova* et al. *(2010).*

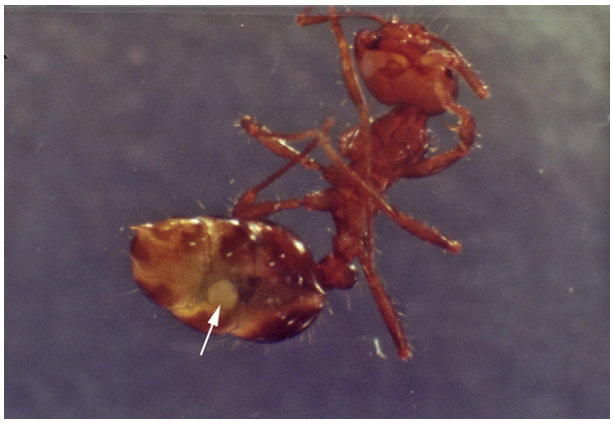


FIGURE 7.8 Cyst (arrow) visible though the cuticle of a red imported fire ant infected with *Kneallhazia* (*Thelohania*) *solenopsae*. (*Photo courtesy USDA-ARS*)

of 88−100% and the demise of *S. invicta* colonies in 6 to 12 months. Queens from infected colonies were debilitated, exhibiting reductions in weight and oviposition (Fig. 7.9), as well as dying prematurely (Williams *et al.*, 1999; Oi and Williams, 2002). Reductions also have been documented in *K. solenopsae*-infected fire ant populations in the field. In Argentina, for example, reductions as high as 83% have occurred in the black imported fire ant, *Solenopsis richteri*, and reductions of 63% have been reported for *S. invicta* in the USA (Briano *et al.*, 1995b; Oi and Williams 2002). Reductions were generally due to decreases in colony size, less brood and fewer workers per nest, and population levels fluctuated (Cook, 2002; Oi and Williams, 2002; Fuxa *et al.*, 2005a). The slow demise of laboratory colonies suggested that field declines could be masked by the immigration or founding of new colonies.

FIGURE 7.9 *Solenopsis invicta* queen infected with *Kneallhazia solenopsae* (right) is smaller than the uninfected queen (left). (*Photo courtesy USDA-ARS*)

Inoculations of K. solenopsae in fire ant populations and surveys for natural infections in the USA revealed that the host's social form and associated behaviors have a profound effect on the prevalence of the pathogen. This microsporidium is found mainly in polygyne *S. invicta*, which is one of the two social forms of fire ants. Monogyne colonies have a single queen per colony and are territorial, thus they fight with other *S. invicta* colonies. In contrast, polygyne colonies are not territorial, and queens, workers, and brood are moved between colonies. The polygynous characteristics extend the persistence and spread of *K. solenopsae* among polygyne colonies (Oi *et al.*, 2004; Fuxa *et al.*, 2005b). The persistence of *K. solenopsae* in polygyne colonies may partly be attributed to the prolonged survivorship of infected colonies afforded by having multiple queens per colony (Williams *et al.*, 1999; Oi and Williams, 2002). Queen infections within a colony are not simultaneous; infection rates of queens from field-collected polygyne colonies ranged from 25 to 75% per *S. invicta* colony (Oi and Williams, 2002). Asynchrony of queen infections is likely to result in a slower demise of colonies because declines in brood production and queen death are staggered. In addition, unlike monogyne colonies, new queens can be adopted by polygyne colonies (Glancey and Lofgren, 1988; Vander Meer and Porter, 2001), providing another source of queens that can prolong the persistence of infected colonies. Polygyny also facilitates the spread of infections given that intercolony movement of infected brood and queens can initiate new colony infections. In contrast, infections in monogyne populations are infrequent in the USA, and when infections do occur they usually dissipate (Fuxa *et al.*, 2005a, b; Milks *et al.*, 2008). In Argentina, *K. solenopsae* infections were found to be nearly equally present in both social forms of *S. invicta*. Perhaps an intermediate host or a more pathogenic strain of *K. solenopsae* contributed to the observed difference in South America (Valles and Briano, 2004).

As indicated, the chronic debilitation of *K. solenopsae*-infected queens suppresses their reproductive capacity. The pathogen also impairs colony founding by infected queens, possibly because of reduced lipid reserves (Cook *et al.*, 2003; Oi and Williams, 2003; Overton *et al.*, 2006; Preston *et al.*, 2007). The abundance of uninfected colonies and the persistent survivorship of infected polygyne colonies, however, often diminish the perceived impact the pathogen is having on field populations. When an area is cleared of fire ants using insecticides, it is normally reinfested within months by colonies migrating from the surrounding area and colonies established by queens that land after mating flights (Collins *et al.*, 1992). Establishment of biological control agents such as *K. solenopsae* in untreated, or unmanaged, landscapes has been used to slow the

reinfestation of landscapes cleared of fire ants. Reinfestations have been delayed by over a year where *K. solenopsae* and fire ant parasitic flies were released and established (Oi *et al.*, 2008). The classical biological control approach of establishing a natural enemy and relying on natural proliferation and spread is potentially the most sustainable method of suppressing well-established invasive species like fire ants which dominate diverse swaths of natural, agricultural, and urban habitats. Of the natural enemies used against fire ants, *K. solenopsae* has the most documented colony-level impacts. To date, *K. solenopsae* has not been found on other continents more recently invaded by fire ants (Yang *et al.*, 2010).

Regional epizootics in both social forms are probably needed to maintain fire ant population suppression. That monogyne fire ants are also commonly infected in South America may signal the possibility of the pathogen being vectored. Several species of fire ant parasitic phorid flies in the genus *Pseudacteon* have been introduced from South America and established in the USA. *Kneallhazia solenopsae* has been detected in at least three species of these flies that develop in infected fire ants; however, there is not yet evidence that the flies can vector the microsporidium (Oi *et al.*, 2009; Oi, unpubl. data).

Kneallhazia solenopsae was reported to be host specific to fire ant species in the *Solenopsis saevissima* species group based on field surveys and laboratory inoculations (Oi and Valles, 2009). However, infections recently have been confirmed in the tropical fire ant, *Solenopsis geminata*, and the *S. geminata* × *Solenopsis xyloni* hybrid, which are in the *Solenopsis geminata* species group. Analysis of 16S ribosomal RNA gene sequences in *K. solenopsae* revealed 22 haplotypes that grouped into two distinct clades. The molecular diversity and expanded host range suggest the presence of variants of *K. solenopsae* with different pathogenicities and host specificity (Ascunce *et al.*, 2010), which may perhaps include different affinities between the fire ant social forms. With the discovery of *K. solenopsae* in the USA, quarantine restrictions were eased and spurred the aforementioned interest and study of its biology and use as a biocontrol agent.

Vairimorpha invictae

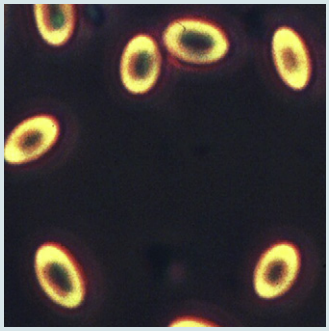
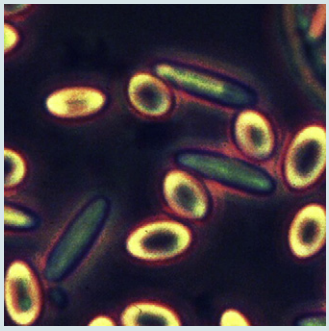
Vairimorpha invictae is another microsporidian pathogen of fire ants from South America that exhibits potential as a microbial biocontrol agent. It was apparently first observed in an infected colony from Brazil a few years after the observation of *K. solenopsae* (Jouvenaz *et al.*, 1980). It has unicellular meiospores that develop in groups of eight within sporophorous vesicles and, in addition, has binucleate, free (not vesicle bound) spores (Jouvenaz and Ellis, 1986). Free spores are bacilliform and develop before the meiospores. They are present as mature spores in larvae, pupae, and adult fire ants. Meiospores are ovoid with a slight narrowing at the anterior end. They begin development in pupae, but mature spores typically are seen in adult ants. Infections were found in all castes of *S. invicta*. Both spore types are larger than *K. solenopsae* spores and have an amber coloration internally under phase-contrast microscopy of unstained specimens (Jouvenaz and Ellis, 1986; Briano and Williams, 2002) (Table 7.3).

Vairimorpha invictae develops in fat bodies located throughout the ant, transforming infected cells into large sacs filled with both spore types (Jouvenaz and Ellis, 1986). Vegetative stages were observed in all immature stages, but they were most frequently seen in fourth instars and pupae. The presence of either vegetative stages or spores in queens and eggs is rare, suggesting inconsistent transovarial transmission (Briano and Williams, 2002). *Vairimorpha invictae* infections can be initiated in colonies with the introduction of live, infected brood. However, unlike *K. solenopsae*, dead *V. invictae*-infected adults have been used successfully as inocula to infect colonies (Oi *et al.*, 2005). The spore type(s) and mechanism involved in intracolony and intercolony infection are not yet known. Infection by isolated spores has not been accomplished, but larvae can become infected when reared to the pupal stage by infected adult workers (Jouvenaz and Ellis, 1986; Oi *et al.*, 2010).

The pathogenicity of *V. invictae* was indicated in laboratory studies where there was a higher prevalence of infection among dead workers than in live workers from diseased *S. invicta* colonies. In addition, mortality occured sooner among naturally infected, starved adult workers than uninfected starved workers (Briano and Williams, 2002). Significant reductions of more than 80% in colony growth were documented after infecting small laboratory colonies of *S. invicta* with live infected brood or dead infected adults (Oi *et al.*, 2005). In Argentina, declines of 69% in field populations of *S. invicta* were associated with natural *V. invictae* infections and also with *K. solenopsae* infections in the same colony (Briano, 2005). Colony declines were faster in the laboratory when infections of the microsporidia occurred together (Williams *et al.*, 2003). The prevalence of dual infections in field colonies has been reported to be about 2% among various sites in South America. The prevalence of *K. solenopsae* and *V. invictae* in field colonies was reported to be 8−13% and 2−10%, respectively, in South American surveys (Briano *et al.*, 1995b, 2006; Briano and Williams, 2002).

The persistence of *V. invictae* field infections appears to be more sporadic, with wide and abrupt fluctuations in prevalence that typically include periods of no infection, whereas infections of *K. solenopsae* were sustained and maintained at fluctuating levels. Peaks of prevalence for the two microsporidian pathogens generally did not coincide, resulting in periods of constant and high disease pressure.

TABLE 7.3 *Vairimorpha invictae* **Spore Types and Size, Primary Fire Ant Stages and Castes, Spore Images, and Descriptions**

Spore Type	Size (μm)	Primary Host Stages and Castes (Occasional Host)	Spore Image[c]	Image Descriptions
Uninucleate meiospores	6.3 × 4.2[a]	Adults of all castes (pupae)[b]		Meiospores without sporophorus vesicle
Binucleate free spores	11.2 × 3.1[a]	Adults of all castes, pupae, larvae[b]		Bacilliform free spores and ovoid meiospores

[a]*Jouvenaz and Ellis (1986);*
[b]*Briano and Williams (2002).*
[c]*Images by J. Briano.*

Using a classical biological control approach with these microsporidia against fire ants would be beneficial for long-term population suppression (Briano *et al.*, 2006). Host specificity testing and field surveys are also supportive of *V. invictae* as a biological control agent. This microsporidium only infects fire ants in the *Solenopsis saevissima* species group, which includes only the invasive *S. invicta* and *S. richteri* in the USA (Briano *et al.*, 2002; Porter *et al.*, 2007; Oi *et al.*, 2010).

The co-occurrence of microsporidia and other natural enemies (Valles *et al.*, 2010) could provide enough diversity in pathogenicity, transmission, and persistence to achieve the goal of self-sustaining suppression of the ubiquitous, invasive fire ant. There are documented laboratory colony declines and population-level field reductions with *K. solenopsae* and *V. invictae*, but sustained reductions perceivable by the public are not consistent in the USA, with only *K. solenopsae* present. *Vairimorpha invictae* is not yet approved for release in the USA, and additional challenges of mass production and efficient inoculation protocols must be developed and implemented. A major biological hurdle is to understand the mechanism(s) of transmission to facilitate consistent infections of one or preferably both microsporidia into monogyne populations in the USA. This would limit reinfestations and slow the further spread of fire ants. Despite the challenges, classical biological control using microsporidia represents a key route toward successfully combating well-established invasive pest ants.

7.4.6. Control of Forest Insect Pests

Compared to most agricultural settings, forests are highly complex ecosystems that, whether old growth or secondary, are vestiges of natural systems with the potential to be harmed by broad-scale chemical inputs. The level of risk to non-target invertebrate forest species posed by chemical insecticides, including chitinase inhibitors such as diflubenzeron that have non-selective toxicity to insects (Eisler, 2000), is controversial (Perry *et al.*, 1997; Beck *et al.*,

2004), and some government entities have severely limited their use, particularly in environmentally sensitive areas. Development of methods to suppress outbreaks and invasive species while avoiding collateral damage to populations of native species is critical for natural systems. When microsporidia are important, host-specific components of the natural enemy complex of exotic forest pests, consideration for introduction as classical biological control agents or augmentative introduction is warranted. Naturally occurring microsporidian pathogens of several forest pests have been studied for their impacts on host populations, and several species of microsporidia infecting the gypsy moth have been extensively evaluated for classical and augmentative introduction for biological control of this serious defoliator.

Impacts of Naturally Occurring Microsporidia on Forest Pests

Microsporidia have been isolated from a number of economically important forest insect species, and it is likely that this pathogen group is far more common than published reports indicate. Solter *et al.* (2010), for example, recovered microsporidia from 23 lepidopteran species collected in two sites in Slovakia (approximately 2000 m^2 total area), although one generalist pathogen, a *Cystosporogenes* sp., represented the majority of the infections (see Section 7.3.7). Microsporidia were also recovered from three species of curculionid bark beetles collected in a one-season survey in Bulgaria (Takov *et al.*, 2011). Microsporidian epizootics are seldom as obvious as those of viruses and fungi, and the role of microsporidia in the population dynamics of their hosts is less well documented. However, a few species are known to be important mortality factors, particularly in species of forest Lepidoptera. Characterizations and descriptions of microsporidia infecting terrestrial forest insect species typically have been based on light microscopy and host relationships, but more detailed documentation that includes biology, molecular data, and epizootiology is available for a few species infecting important pests (Table 7.4). Although acute mortality caused by microsporidiosis is not common, the sublethal effects detailed in this chapter may strongly affect populations of many forest insects (Gaugler and Brooks, 1975; Wilson, 1980), and several species have been evaluated for biological control programs.

Spruce Budworm Microsporidia

Four species of microsporidia have been reported infecting the spruce budworm, *Choristoneura fumiferana*: *Cystosporogenes* sp., *Endoreticulatus* (*Pleistophora*) *schubergi*, *N. fumiferanae* and *Thelohania* sp. (van Frankenhuyzen *et al.*, 2004). Most of these microsporidia are encountered infrequently, but *N. fumiferanae*, a species closely related to the microsporidian type species, *N. bombycis* (Kyei-Poku *et al.*, 2008), is common and often occurs at high prevalence levels, increasing in spruce budworm populations as larvae mature over the summer months (Wilson, 1973) and over the years that host populations persist at high densities (Thomson, 1960; Wilson, 1973). In one spruce budworm population, prevalence increased from 36% to 81% over a five-year period (Thomson, 1960) and, 17 years later, increased from 13% to 69% over a six-year period in the same site (Wilson, 1977). The spruce budworm population declined in the years following the peak prevalence (Thomson, 1960).

A fine-scale model of spruce budworm population dynamics that included *N. fumiferanae* infection as an independent variable showed that the microsporidium increased mortality of spring-emerging larvae (Régnière and Nealis, 2008). The model was corroborated in studies showing that transovarial transmission of *N. fumiferanae* by intensely infected spruce budworm females in older outbreak populations usually produces 100% infected offspring, resulting in reduced survival during dormancy, delayed emergence from hibernacula and dispersal, reduced establishment on feeding sites and, based on prevalence studies, high mortality between the period of dispersal and establishment in feeding sites (van Frankenhuyzen *et al.*, 2007). In a study of western spruce budworm, *Choristoneura occidentalis*, males infected with a closely related *Nosema* sp. (Kyei-Poku *et al.*, 2008) were less likely to fly upwind to a pheromone source, suggesting that infection reduces mating success in infected populations (Sweeney and McLean, 1987).

Both *N. fumiferanae* and another microsporidium, *E. schubergi*, were evaluated in the laboratory and field as potential microbial insecticides. Laboratory studies conducted to evaluate transmission and effects of the two species showed reduced pupal weight, fecundity, and adult longevity (Thomson, 1958; Wilson, 1984) in orally infected hosts, as well as high larval mortality for F1 larvae transovarially infected by *N. fumiferanae* (Bauer and Nordin, 1989a). Bauer and Nordin (1989b) determined that both LC_{50} and LT_{50} of *B. thuringiensis* treatments were lower for infected spruce budworm than for uninfected hosts, but LC_{50} values observed in larvae from multiple spruce budworm populations were not affected when natural prevalence of *N. fumiferanae* was not augmented in the laboratory (van Frankenhuyzen *et al.*, 1995).

Sprayed on trees to increase prevalence in host populations, levels of *N. fumiferanae* remained higher in treated sites than in untreated sites for several years until levels of natural infections rose to similar levels, suggesting that augmentation successfully advanced epizootics by two to three years (Wilson and Kaupp, 1976). *Endoreticulatus schubergi* persisted at low levels; it was hypothesized that

TABLE 7.4 Partial List of Microsporidia Infecting Forest Insect Pests

Host Species	Microsporidian Species	References[a]
Agrilus anxius	*Cystosporogenes* sp.	Kyei-Poku *et al.* (2011)
Archips cerasivoranus	*Endoreticulatus* (*Pleistophora*) *schubergi*	Wilson and Burke (1978)
Choristoneura conflictana	*Nosema thomsoni*	Wilson and Burke (1971)
Choristoneura fumiferana	*Cystosporogenes* sp. (*legeri?*)	van Frankenhuyzen *et al.* (2004)
	Endoreticulatus (*Pleistophora*) *schubergi*	Wilson (1975)
	Nosema fumiferanae	Thompson (1955)
	Thelohania sp.	Wilson (1975)
Dendroctonus species	*Nosema dendroctoni*	Weiser (1970)
	Chytridiopsis typographi	Knell and Allen (1978)
	Unikaryon minutum	
Euproctis chrysorrhoea	*Nosema chrysorrhoeae*	Hyliš *et al.* (2006)
	Nosema kovacevici	Purrini and Weiser (1975)
	Endoreticulatus sp.	Purrini (1975)
Hyphantria cunea	*Endoreticulatus schubergi hyphantriae*	Weiser (1961)
	Nosema sp. (*bombycis*-type)	Solter and Maddox (1998b)
	Vairimorpha sp.	
Ips spp.	*Chytridiopsis typographi*	Purrini and Weiser (1985)
	Larssoniella duplicati	Weiser *et al.* (2006)
	Nosema typographi	Weiser *et al.* (1997)
	Unikaryon montanum	Wegensteiner *et al.* (1996)
Lymantria dispar	*Endoreticulatus schubergi*	McManus and Solter (2003)
	Nosema lymantriae	
	Nosema portugal	
	Nosema serbica	
	Vairimorpha disparis	
Malacosoma americanum	*Nosema* sp. (*bombycis*-type)	Weiser and Veber (1975)
Malacosoma disstria	*Nosema disstriae*	Thomson (1959)
Operophtera brumata	*Cystosporogenes operophterae*	Canning *et al.* (1983)
	Nosema wistmansi	
	Orthosoma operophterae	
Pristiphora erichsoni	*Thelohania pristiphorae*	Smirnoff (1967)
Tomicus piniperda	*Caningia tomici*	Kohlmayr *et al.* (2003)
Tortrix viridana	*Nosema tortricis*	Franz and Huger (1971)

[a] *References are species descriptions or research reports.*

low prevalence was due to mortality in the year of treatment, but no mortality studies were conducted.

Microsporidian Pathogens of Gypsy Moth

The gypsy moth, a defoliating outbreak species that was introduced to North America in the late 1860s, is a generalist feeder with preference for oaks (*Quercus* spp.) and aspens (*Populus* spp.) in North America (Leonard, 1981). Although the adult female does not fly, natural spread by ballooning neonate larvae and, more importantly, inadvertent human transport of egg masses (Allen *et al.*, 1993) have served to advance the movement of the pest from the site of introduction in Massachusetts to the current western leading edge in Wisconsin and north-eastern Illinois and south to Virginia, North Carolina, and Kentucky.

Nearly a century of attempts to control the gypsy moth resulted in a federal government, private industry, and state agency partnership in the USA to produce a comprehensive insect pest management effort. The "Slow the Spread of Gypsy Moth" (STS) program, established in 1999 and spearheaded by the USDA Forest Service, uses intensive monitoring and control, primarily with the microbial insecticide *B. thuringiensis*, to eradicate isolated populations that occur in advance of the leading edge of invasion, as well as treatment of outbreak populations at the leading edge and elsewhere in the infested zones. A cornerstone of the program is the use and manipulation of naturally occurring pathogens to suppress populations in the area of establishment. Solter and Hajek (2009) reviewed the research on several microsporidian species isolated from gypsy moth populations, as well as studies of other pathogens, parasites, and predators that have been manipulated as biological control agents of the gypsy moth. There follows a brief summary of the program and the studies leading up to release of microsporidia as augmentative and classical biological control agents.

Naturally occurring pathogens have been extensively incorporated in the gypsy moth STS program; two of them, nucleopolyhedrovirus, *Ld*MNPV (Chapter 4) and the fungal pathogen *Entomophaga maimaiga* (Chapter 6), have potential impacts on introductions of microsporidia. *Ld*MNPV was probably accidentally introduced into North America with the pest and is currently formulated by the USDA Forest Service as a host-specific microbial pesticide, Gypcheck® (Podgwaite, 1999). Produced in limited quantities, it is a particularly useful biopesticide in environmentally sensitive areas (Reardon *et al.*, 2009). The introduction from Asia, probably Japan (Nielsen *et al.*, 2005), of the host-specific *E. maimaiga* is an inadvertent success story. Whether the introduction was purposeful or accidental has not been determined (Solter and Hajek, 2009), but *E. maimaiga* is now well established in the USA and is currently present and frequently causes epizootics in gypsy moth populations on the leading edge of invasion (Villidieu and van Frankenhuyzen, 2004; L. F. Solter, unpubl. data). *Entomophaga maimaiga* has been introduced in eastern Europe and is now established in Bulgaria and Macedonia (Pilarska *et al.*, 2000, 2007).

Although several microsporidian species are commonly present in gypsy moth populations in Europe, evidently none was present in the gypsy moth population that first invaded US forests in the late 1860s; they have never been recovered from North American gypsy moth field populations (Campbell and Podgwaite, 1971; Podgwaite, 1981; Andreadis *et al.*, 1983; Jeffords *et al.*, 1989). There are seven species descriptions for microsporidia pathogenic to gypsy moth in the literature (Solter and Hajek, 2009). Of those, five species are currently recognized: *E. schubergi*, *N. lymantriae*, *V. disparis* (= *Thelohania disparis*, = *Thelohania similis* + *N. lymantriae*), *Nosema serbica* (known only from stained slides), and *N. portugal.* Vávra *et al.* (2006) disentangled the early species descriptions in the literature and recharacterized *V. disparis*; however, it is still not clear whether the named species in the *Nosema* group are biotypes of the same species or should retain species status.

Microsporidia were first reported to be important factors in gypsy moth population declines in central and eastern Europe, including Serbia (Sidor, 1976) and the Ukraine (Zelinskaya, 1980), where the gypsy moth is native. Particularly noted was the precipitous decline of populations with high prevalence of both microsporidia and *Ld*MNPV. Indeed, synergy between the virus and *N. portugal*, based on mortality rate, was shown for mixed infections in laboratory bioassays, particularly when *N. portugal* was orally inoculated before inoculation with the virus (Bauer *et al.*, 1998).

The first efforts to manipulate the pathogens for gypsy moth control were augmentative field experiments with *N. lymantriae* in Slovakia. A 79% increase in mortality was recorded in the site treated with *N. lymantriae* foliar sprays by the time adults eclosed and numbers of progeny were reduced by 90% (Weiser and Novotny, 1987). Novotny (1988) reported that artificial declines in gypsy moth populations were nearly equally precipitous for plots treated with *Ld*MNPV and those treated with *N. lymantriae.* In a 1986 study in the USA, two species, *N. portugal* and *E. schubergi*, were released in isolated 4 ha (10 acre) woodlots in Maryland by attaching gypsy moth egg masses soaked in spore suspensions to oak trees. *Endoreticulatus schubergi* was not recovered the following year, but *N. portugal* overwintered and was horizontally transmitted during the 1987 season (Jeffords *et al.*, 1989), indicating that it could become established. *Nosema portugal* apparently did not persist; it was not recovered in a 1989 field collection (M. Jeffords, LFS, unpubl. data). A second attempt in Michigan to release *N. portugal* via spore-saturated egg

masses failed, perhaps owing to loss of viability resulting from overlong exposure of the spores to environmental conditions before hatch (Solter and Becnel, 2007).

Beginning in the mid-1990s, laboratory and field experiments were focused on determining mechanisms of transmission, and on safety to non-target organisms. As with other microsporidian species, transmission is related to the tissue specificity of the pathogen. *Endoreticulatus schubergi*, strictly a midgut parasite, is spread via infective spores in the feces of the host. In contrast, *V. disparis*, primarily a fat body pathogen, has little egress from the host except when infections are very intense and persist for over two weeks, in which case, the Malpighian tubules become infected and some spores are present in the feces. More typically, *V. disparis* is transmitted by degradation of dead infected hosts in the environment (Goertz and Hoch, 2008a, b). *Nosema lymantriae* and *N. portugal* are more systemic, maturing in the silk glands (Fig. 7.10), fat body tissues, Malpighian tubules, and gonads. Environmental spores were found in the silk of *N. portugal* and it was hypothesized that the pathogen could be transmitted on silk trails (Jeffords *et al.*, 1987), but Goertz *et al.* (2007) found little evidence that the silk served as a conduit for infective *N. lymantriae* spores among susceptible larvae. *Nosema lymantriae*, like *V. disparis*, is probably spread via decomposing infected hosts, but, after a latency period, is also transmitted via the feces at an increasing rate over the course of infection (Goertz and Hoch, 2008a), and is transovarially transmitted from infected female to the offspring (Goertz and Hoch, 2008b). Gonads of the gypsy moth host are also infected by *V. disparis*, but this species is a more virulent pathogen and kills its host before eclosion, no matter the dosage or age at inoculation (Goertz and Hoch, 2008b). Field cage experiments quantified horizontal transmission of *N. lymantriae* and also demonstrated the latency period for this microsporidium (Hoch *et al.*, 2008).

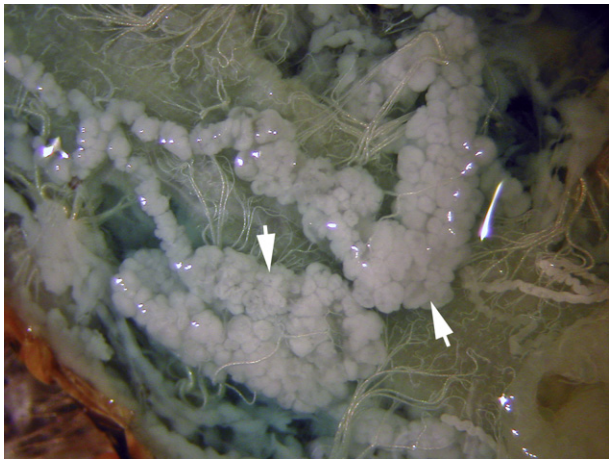


FIGURE 7.10 Gypsy moth silk glands (arrows) infected with *Nosema lymantriae*. (*Photo by G. Hoch.*)

Test larvae acquired infections nearly three weeks after exposure to newly inoculated treated larvae.

Solter *et al.* (1997) tested 50 species of North American forest Lepidoptera for susceptibility to *N. portugal*, two isolates of *N. lymantriae*, *V. disparis*, and *E. schubergi.* All isolates were infective to some of the lepidopteran species orally inoculated in the laboratory, and *E. schubergi* infections were often patent, but atypical *V. disparis* and *N. lymantriae* infections observed in many non-target hosts suggested that the pathogens would not reproduce optimally and would not be transmitted. Using gypsy moth as a model non-target host, Solter and Maddox (1998b) determined that non-target infections (microsporidia of native North American forest Lepidoptera infective to gypsy moth) were not horizontally transmitted among gypsy moth larvae. In addition, field studies in Bulgaria and Slovakia found no Lepidoptera sympatric with gypsy moth to be infected with naturally occurring gypsy moth microsporidia (Solter *et al.*, 2000, 2010). Inundative sprays of *V. disparis* in Slovakia (Solter *et al.*, 2010) caused apparently "dead-end" *V. disparis* infections in limited numbers of several non-target species; some infections were atypical, and no microsporidia were found infecting the susceptible species in subsequent years. Only one non-target infection was found where *N. lymantriae* was sprayed. Based on host specificity studies, US regulatory agencies permitted releases of Bulgarian isolates of *V. disparis* and *N. lymantriae* in 2008 and 2010. Studies of releases into rising natural gypsy moth populations in Illinois and in naïve Bulgarian populations are ongoing (L. F. Solter, D. K. Pilarska, and A. Linde, unpubl. data).

7.4.7. Microsporidia Infecting Biological Control Agents

The introduction of biological control agents to a new system is a complicated process encompassing a range of issues including host specificity, mass production, establishment in the field, and efficacy of control of the target pest. Another potential layer of complexity includes accidental importation of natural enemies of the agent as "contaminants" (Goettel and Inglis, 2006). While the more easily observed predators and parasites of a putative biological control agent usually can be eliminated during a quarantine period, pathogens may be much more difficult to detect, particularly if prevalence is initially low and effects are chronic, all typical for microsporidian infections. Unless obvious signs of disease occur in quarantined insects, pathogens arriving with their hosts may only begin to cause noticeable problems when mass rearing operations are established. High density and stresses on mass-reared insects can exacerbate disease transmission, resulting in unusually high prevalence and mortality rates (Goodwin, 1984), and infection can result in the loss of entire colonies

(Bhat *et al.*, 2009; Solter *et al.*, in press). A pathogenic organism released into the environment by way of infected biological control agents can negatively impact the release effort owing to debilitation and excessive mortality of the agent. There is also a risk that an exotic pathogen infecting the biological control agent can invade populations of closely related hosts in the field.

Goettel and Inglis (2006) suggested that the risk posed by pathogens of invertebrate biological control agents is greater for invertebrates collected from the field for direct release against a pest than for mass-reared agents that have been observed and screened for pathogens, and that exotic invertebrates intended for release as classical biological control agents may pose a higher risk than indigenous agents that are used in inundative biological control programs. Nevertheless, inundative releases of mass-reared ubiquitous (augmentative) agents may be problematic if epizootics occur during mass production and populations of conspecific naturally occurring predators and parasitoids are inadvertently exposed to the pathogen, as described for local bumble bee populations where commercially reared bees are used as pollinators (Colla *et al.*, 2006; Otterstatter and Thomson, 2008).

Goeden and Louda (1976) and Bjørnson and Schütte (2003) presented detailed discussions about the effects of microsporidia and other pathogens infecting a variety of insects reared for use in biological control programs. A *Nosema* sp., probably *Nosema tyriae* (Canning *et al.*, 1999), for example, was cited as significantly reducing the effectiveness of the arctiid *Tyria jacobaeae* in a biological control program for tansy ragwort (Harris *et al.*, 1971; Hawkes, 1973), and the pyralid *Nosema cactoblastis* compromised field releases and insectary rearing of the cactus moth *Cactoblastis cactorum* in control programs for prickly pear cactus (Pettey, 1947). Microsporidian disease reduced the fecundity and predation activity of predatory mites, including *Amyblyseius* spp. (Van Der Geest, 2000; Bjørnson and Schütte, 2003) and *Phytoseiulus* spp. (Bjørnson and Keddie, 1999). In addition to these reports, Solter *et al.* (in press) recovered five microsporidian species from four species of coccinellid and derodontid beetles being mass reared for biological control of the hemlock woolly adelgid, *Adelges tsugae* (Reardon and Onken, 2004). Three of the species caused high mortality in the colonies and resulted in loss of mass-reared insects for the release effort. One species, isolated from the coccinellid *Sasajiscymnus tsugae*, produced heavy infections and mortality in three other species of predatory beetles being reared for the control effort, raising concerns that high infection prevalence in *S. tsugae* released in or near sites where other beetle species were released could compromise those release efforts.

The eradication of all pathogens is probably impossible for any insect rearing situation, particularly those that do not have obvious symptoms or cause acute mortality and are, thus, difficult to detect. The *A. tsugae* study and those previously reported (Bjørnson and Schütte, 2003; Goettel and Inglis, 2006), however, highlight the importance of determining whether deleterious infectious diseases are present in putative biological control agents and eliminating pathogens before full-scale mass production as initiated.

7.5. FUTURE RESEARCH DIRECTIONS

Microsporidia are important as regulators of natural insect populations and have potential as classical and augmentative biological control agents of insect pests. Species infecting beneficial and economically important insects are also potentially harmful if accidentally introduced to new habitats or to managed insect populations and colonies. Key areas of basic research needed include increased understanding of host−parasite interactions such as mechanisms of infection, transmission, and host specificity. Tissue specificity, host immune response, and evasion of immune response should be elucidated at the cellular and molecular levels, and model systems designed to better understand the ecology of microsporidian parasites from different host and habitat types.

Genome sequences of a diversity of microsporidian entomopathogens, together with those of their hosts, are essential to understanding gene function and interactions at the protein level, as well as contributing to a better understanding of phylogenetic relationships within and between species with invertebrate and vertebrate hosts. Taxonomic data, including identity of strains, will become increasingly important information to collect for pathogens under consideration for use in biological control programs.

The effects on host fitness caused by multiple parasitism, infection of individual hosts and host populations by multiple pathogen and parasite species, is also an important area of study. Unexpected and confounding results of introducing biological control agents may be explained and failures avoided if the antagonistic, additive, or synergetic effects of multiple parasitism are known and accounted for. In addition, multiple pathogens and parasites can cause major problems with economically important managed insects; declines in honey bee populations, for example, have been hypothesized to be related to high loads of multiple pathogen and parasite species in high-density, heavily managed apiaries.

Several extensive efforts to use microsporidia as classical biological control agents in a variety of habitats have been undertaken. They appear to be most important in systems where other compatible natural enemies are present, where transmission potential is high, where suppression rather than eradication is the goal, and where immediate control of pests is not required. Efficacy in aquatic systems is confounded by the frequent necessity for

fast-acting control measures, inconsistent persistence of the pathogens and hosts, and concerns about pathogens of mosquitoes, which take blood meals from humans and other vertebrate animals. Evidence that some mosquito-infecting microsporidia or closely related species can or do infect humans, albeit infrequently, has resulted in eliminating some species from consideration as biological control agents. Nevertheless, augmentative releases of some microsporidian species into naïve host populations is a viable method of increasing the load of natural enemies for long-term control of pest aquatic insects.

The situation is similar for microsporidia of terrestrial pests; for short-term control needed in most agricultural settings, microsporidia are not sufficiently virulent to act as microbial insecticides. Their best use is as augmentative or classical biological control agents in less intense settings, e.g., forests, rangelands, orchards, and prairies. *Paranosema locustae* has arguably had the most significant impact by controlling grasshoppers in the rangeland ecosystem (Lange, 2005; Shi *et al.*, 2009). High virulence and abundant, easily dispersed spores all contribute to its success, and it is still sold in North America as a "garden use" microbial insecticide. The inherent chronic effects caused by most microsporidian infections, however, have prevented declarations of completely successful biological control using these unique pathogens. In general, completely successful biological control requires a complex of diverse agents, and certainly microsporidia can and do play a part in suppression of many insect species. To enhance the utilization and impact of microsporidia, future efforts to characterize microsporidia and their interactions with their hosts at all levels will aid in the discovery of exceptionally adapted strains or cryptic species for biological control.

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