Journal of Invertebrate Pathology 151 (2018) 113–125

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Amblyospora khaliulini (Microsporidia: Amblyosporidae): Investigations on its life cycle and ecology in *Aedes communis* (Diptera: Culicidae) and *Acanthocyclops vernalis* (Copepoda: Cyclopidae) with redescription of the species

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ARTICLE INFO

Keywords:
Microsporidia
Amblyospora khaliulini
Life cycle
Morphology
Transmission
Ultrastructure
Epizootiology
Mosquito
Aedes communis
Copepod
Acanthocyclops vernalis

ABSTRACT

A multi-year study was conducted to examine the natural ecology of the microsporidium *Amblyospora khaliulini* and more fully characterize parasite development and histopathology in all stages of its primary mosquito host, *Aedes communis* and intermediate copepod host, *Acanthocyclops vernalis* with redescription of the species. *A. khaliulini* exhibits polymorphic development, produces three morphologically and functionally distinct spores, and is both horizontally and vertically transmitted. Development in *A. vernalis* is restricted to females, occurs within the ovaries and results in death of the host. Development is haplophasic with division by binary and multiple fission producing rosette-shaped sporogonial plasmodia and conical uninucleate spores that are orally infectious to *Ae. communis* larvae. Both sexes are equally susceptible and infections are confined to testes in males and ovaries in females. Initial stages of development include uninucleate schizonts that undergo karyokinesis forming diplokaryotic meronts that divide repeatedly by binary fission. Sporogony occurs in both host sexes, but sporogenesis does not progress normally in adult males and elliptical, thin walled binucleate spores that function in vertical transmission of the microsporidium via infection of the ovaries and eggs are formed in adult females only. Development of vertically acquired infections in larval *Ae. communis* hosts occurs within fat body tissue, leads to the production of meiospores in male hosts only and results in death during the 4th larval stadium. Initial development is characterized by merogonial multiplication of diplokarya by synchronous binary division producing additional diplokarya. The cessation of merogony and the onset of sporogony are characterized by the simultaneous secretion of a sporophorous vesicle and meiotic division of diplokarya resulting in the formation of octonucleate sporonts that undergo cytokinesis and sporogenesis to form eight uninucleate, broadly ovoid meiospores enclosed within a sporophorous vesicle. The natural prevalence of patent vertically acquired fat body infections in field populations of *Ae. communis* ranged from 1.6% to 3.6%. Yearly infection rates in *A. vernalis* copepods ranged from 57.1% to 15.0%. Prevalence rates of horizontally acquired infections in emerging adult *Ae. communis* ranged from 69.0% to 11.9% in males and 50.0% to 16.4% in females.

1. Introduction

Amblyospora khaliulini Hazard and Oldacre, 1975 is a little known microsporidian parasite of the univoltine, boreal mosquito, *Aedes communis* (DeGeer) (Hazard and Oldacre, 1975). First recognized in 1920 as a species of *Thelohania* from a few patently infected larval mosquitoes with “white cysts” collected in Germany (Noller, 1920), it was similarly reported from late stage larval specimens collected in the Czech Republic (*Thelohania opacita*) (Weiser, 1947); the former U.S.S.R (*Thelohania opacita* var. *mariensis*) (Khaliulin and Ivanov, 1971); Manitoba, Canada (*Thelohania* sp.) (Welch, 1960); Alaska (*Thelohania* sp.) (Chapman et al., 1973) and Massachusetts (holotype) (Hazard and Oldacre, 1975) in the United States. Welch (1960) remarked on its gross pathology noting that the parasite typically killed the larval host by infecting fat body tissue and preventing host pupation. He further reported that it was present in a high percentage of forest pools (up to 86%) in Churchill, Manitoba and was responsible for a reduction of 3–11 percent of the larval population. Rudimentary observations on the sporogonic sequence producing “octospores” (now known as meiospores) and spore size were also documented from the larval host, but no attempt was made to identify the source of infection and no other aspects of the life cycle were revealed.

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https://doi.org/10.1016/j.jip.2017.11.007
Received 7 September 2017; Received in revised form 24 October 2017; Accepted 7 November 2017
Available online 08 November 2017

Hazard and Oldacre (1975) subsequently revised the *Thelohania* and established a new genus, *Amblyospora* to include many mosquito-parasitic species with dimorphic development and oval truncated "octospores". *Amblyospora khaliulini* was accordingly assigned and redescribed. However, this was based solely on morphological characteristics and ultrastructure of the spore produced in infected larvae. No further details on parasite development, morphology or pathology in adult hosts, transmission mechanisms or features of the life cycle were identified, nor the involvement of the intermediate copepod host which had yet to be discovered.

While conducting a survey for microsporidia infecting natural populations of mosquitoes inhabiting forested wetlands in the northeastern United States, *A. khaliulini* was recovered from an isolated population of *Ae. communis* inhabiting a semi-permanent, vernal pool in northwestern Connecticut, USA. Accordingly, we initiated a multi-year study to examine the natural ecology of *A. khaliulini* and more fully characterize parasite development and histopathology in all stages of both the mosquito and intermediate copepod hosts by light and electron microscopy with full redescription of the species.

2. Materials and methods

2.1. Study site and field ecology

The study site was an evergreen forest, dominated by eastern hemlock (*Tsuga canadensis*) and eastern white pine (*Pinus strobus*) located in Barkhamsted, Litchfield County, CT, USA (41° 57′ 48″N, 72° 53′ 94″W). The aquatic habitat was a well-defined leaf-lined, vernal pool that was typically dry by June or early July (Fig. 1.). The pool supported populations of two univoltine mosquitoes, *Ae. communis* and *Aedes excrucians* (Walker) and the cyclopoid copepod, *Acanthocyclops vernalis* (Fischer).

Field studies were conducted in 1999, 2000, 2001 and 2005 for the purpose of assessing the natural prevalence of *A. khaliulini* infection in *Ae. communis* and its intermediate host, *A. vernalis* throughout their respective stages of development within the aquatic habitat. Sampling was conducted weekly from the onset of larval mosquito hatch (late March to early April) until pupation (mid to late May). Immature mosquitoes and copepods were collected from the pool with a standard 350 ml mosquito dipper and immediately transported to the laboratory for examination. Mosquitoes were identified using keys and descriptions from Darsie and Ward (1981) and Means (1979). Copepods were identified using keys and descriptions of Yeatman (1959) and Pennak (1989). Confirmation on the identity and conspecificity of *A. khaliulini* from both hosts was corroborated from SSU rDNA sequences obtained from mature spores procured from naturally infected *Ae. communis* larvae and adult female *A. vernalis* (GenBank/EMBL database Accession Nos. AY090045, AY090046, AY090047) (Vossbrinck et al., 2004).

Fig. 1. Vernal pool study site as seen in early April with early melting along perimeter.

The weekly prevalence rate of *A. khaliulini* infection in both mosquito and copepod populations was determined from microscopic (1000x) examination of Giemsa-stained smears (10% solution, pH 6.8) of a minimum of 50 whole mosquito larvae, and up to 50 whole adult female copepods collected on each sample date. Smears were air dried, fixed in 100% methanol (5 min), and stained with a 15% (v/v) modified Giemsa stain solution (pH 7.4) (20 min) (Sigma-Aldrich Accustain® Giemsa Stain, Modified, St. Louis, MO). Larval mosquito development was typically uniform, but in instances where mixed larval instars were collected, equal numbers of each were examined in an effort to obtain a prevalence rate that was representative of the entire population. Only female copepods were examined as prior investigations with other species of *Amblyospora* (Andreadis, 1988a) and *Hyalinocysta* (Andreadis and Vossbrinck, 2002) had shown males to be refractory to infection owing to the site of parasite development in ovarian tissue.

In order to assess infection prevalence in the emerging adult mosquito population, field collected pupae were individually isolated in 30-ml plastic containers, held at room temperature and similarly examined for infection 1–2 days after emergence. Overall prevalence rates were based on examination of an equal number of males and females (up to 50 each). In all instances, individual copepods and mosquitoes were scored as infected if any developmental stage (vegetative or spore) was observed.

2.2. Life cycle studies: Light microscopy

General characterization of microsporidian development in both the mosquito and copepod hosts was made from microscopic examination (1000x) of Giemsa-stained smears of infected tissues obtained from live mosquito larvae, emerging adults and female copepods collected from the field, and from larval *Ae. communis* infected in the laboratory transmission studies (see Section 2.4). Tissue specificity was determined from histological examination of paraffin-embedded whole larval, adult male and female stages of *Ae. communis* and plastic embedded *A. vernalis*. Paraffin sections were stained with iron hematoxylin and eosin Y. Measurements of mature spores were calculated from examination of whole wet-mount preparations of live spores (n = 50) with differential interference optics in a Zeiss Axioplan 2 Digital imaging system (1000x).

2.3. Life cycle studies: Ultrastructure

The comparative ultrastructure of microsporidian development was ascertained from examination of adult female *A. vernalis* and larval and adult male and female stages of *Ae. communis*. Infected tissues were fixed in a 2.5% (v/v) glutaraldehyde solution containing 0.1% (w/v) $CaCl_2$ and 1% (w/v) sucrose buffered in 100 mM Na cacodylate (pH 7.3) overnight at room temperature and postfixed in 1% (w/v) OsO_4 in the same buffer and temperature. Fixed specimens were dehydrated through a graded ethanol and acetone series and embedded in a LX-112/Araldite (Ladd Research Industries, Williston, VT) mixture. Thin sections (60–100 nm) were stained with 5% (w/v) uranyl acetate in 50% (v/v) methanol followed by Reynold's lead citrate, and examined in a Zeiss EM 10C electron microscope at an accelerating voltage of 80 kV.

2.4. Transmission studies

Horizontal transmission studies were conducted in the laboratory to (1) qualitatively assess the oral infectivity of spores of *A. khaliulini* procured from field collected *A. vernalis*, and (2) corroborate the source and type of infection observed in larval populations of *Ae. communis* in

the field. Field collected *Ae. communis* larvae were used in all assays as the species is not colonizable. Each bioassay was performed at 25 °C under a 16:8 (LD) photoperiod in 100 × 80 mm culture dishes containing 100 ml of filtered water (0.45 µm) from the vernal pool collection site. Fifty 3rd instar *Ae. communis* larvae were placed in each culture dish (n = 2) along with two heavily macerated, infected *A. vernalis* copepods each containing approximately 1×10^4 mature spores. A small amount of an aqueous suspension of Brewer's yeast and liver powder (2:3 mixture) was provided for food. Controls consisted of an identical number of larvae that were maintained in separate culture dishes without infected copepods. Larvae were reared for 7 days following which they were individually smeared on microscope slides, stained with Giemsa, and examined microscopically as described in Section 2.1. The type and prevalence of infection was recorded and compared with that observed in field collected specimens.

3. Results

3.1. Development in Acanthocyclops vernalis

The initial developmental stages observed in adult female *A. vernalis* copepods were small (5–10 µm), oval shaped uninucleate schizonts (Fig. 2). These were bound by a simple plasmalemma that was in direct contact with the host cell cytoplasm and contained a homogeneously granular cytoplasm with numerous free ribosomes (Fig. 8). Shizogony appeared to occur by both binary and multiple division (Fig. 2-3). No diplokaryotic stages were observed. The onset of sporogony was characterized by repeated nuclear division forming large (up to 30 µm) multinucleate, lobbed, rosette-shaped plasmodia with up to eight nuclei (Fig. 4-5). These plasmodia were not bound by any apparent sporophorous vesicle (Fig. 9). Cytoplasmic division was by budding of the sporogonial plasmodium forming unicellular sporoblasts (Fig. 5,6 and Fig. 10). Early sporoblasts (Fig. 10) possessed a vacuolated cytoplasm, a network of endoplasmic reticulum and zones of Golgi apparatus.

Mature spores were conical and averaged 11.3 ± 0.4 × 4.4 ± 0.1 µm (live) (mean ± SD) (Fig. 7). They were uninucleate and possessed a large electron lucent posterior vacuole (Fig. 11). The polaroplast was voluminous, occupying the inner two-thirds of the spore and was bipartite consisting of large irregularly spaced vesicular chambers in the anterior end and more tightly compressed lamellar elements in the posterior end. The polar filament was isofilar with 12–15 coils largely arranged in a single row but with notable clustering of the last three most posterior turns (Fig. 11,12). The anchoring disc of the polar sac was well developed (Fig. 13). The exospore was slightly undulating, without ornamentation and measured approximately 20 nm, while the electron lucent endospore measured approximately 100 nm (Fig. 12,13).

Microsporidian development occurred within the median ovary and paired lateral oviducts of the female copepod host. These became grossly distended due to the production of approximately 1×10^4 spores and ultimately resulted in death of the copepod with the rupture of infected tissues (Fig.14).

3.2. Development in Aedes communis: Vertically acquired infections

Field collected larval mosquitoes with patent infections that were subsequently shown to be vertically acquired possessed opaque swollen white masses in the thorax and segments of the abdomen that were readily apparent when viewed through the transparent larval cuticle against a black background (Fig. 15). Infections were restricted to fat-body tissue and were not detected in any other internal tissues. Mortality occurred prior to pupation during the fourth larval stadium. Histological examination of twenty-two heavily infected fourth instar larvae revealed that all were male based on the detection of the sausage-shaped testes and surrounding layer of uninfected fat-body situated in the sixth abdominal segment.

The sequence of microsporidian development in these larval hosts was ascertained through ultrastructural examination of infected tissues. The earliest developmental stages observed were simple meronts (~ 10 µm in length) with nuclei in the diplokaryotic arrangement (Fig. 16). These meronts were delimited by an unadorned plasmalemma in direct contact with the host cell cytoplasm and contained a densely granular cytoplasm with numerous free ribosomes and occasional stacks of endoplasmic reticulum. Meronts appeared to undergo synchronous nuclear division forming transitory merogonial plasmodia with two diplokarya (Fig. 17). This was followed by cytoplasmic division that gave rise to additional diplokaryotic meronts which we interpreted as a proliferative phase of merogony.

The onset of sporogony was marked by a thickening of the cell plasmalemma, the formation of a thin sporophorous vesicle of parasite origin and the accumulation of electron dense granular inclusions within the eipsporontal space (Fig. 18). This was coincident with the onset of meiosis as evidenced by the physical separation of each member of the diplokaryon and partial dissolution of the integrity of the nuclear envelopes, the detection of synaptonemal complexes within each nucleus, and the appearance of a distinct crescent shaped organelle, "kinetic center" within the furrow separating each member of the diplokaryon. Densely stacked arrays of endoplasmic reticulum were additionally prominent within the cytoplasm. Meiosis I resulted in the formation of a binucleate sporont with individual nuclei at each pole and a distinct constricted cell membrane (Fig. 19). During meiosis II, quadrinucleate sporogonial plasmodia were formed by synchronous mitotic division of each nucleus (Fig. 20). This was followed by a final mitotic division producing octonucleate sporogonial plasmodia that subsequently underwent cytokinesis to produce eight uninucleate sporoblasts within the persistent sporophorous vesicle (Fig. 21). Sporogenesis ensued with initial morphological differentiation of the polar filament, followed by the polaroplast and anchoring disc, posterior vacuole, and lastly the endospore and exospore wall (Fig. 22,23).

Mature spores were broadly ovoid and averaged 7.0 ± 0.1 × 4.7 ± 0.1 µm (live) (mean ± SD) (Fig. 15, Fig. 24). They were uninucleate and possessed a large posterior vacuole containing a mesh-like matrix. The polaroplast was bilaminar with more closely packed anterior lamellae and the polar filament was anisofilar with 3–4 broad proximal coils and 11–12 narrow distal coils mostly arranged in a single row (Fig. 23,24). The anchoring disc of the polar filament was

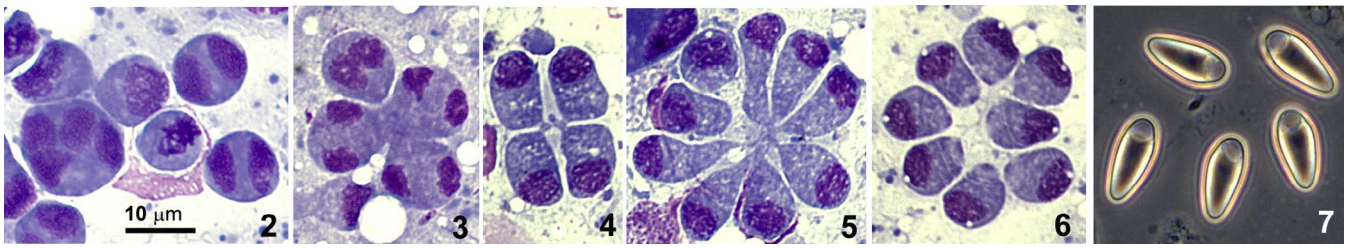

Fig. 2–7. Developmental stages of *Amblyospora khaliulini* as observed in Giemsa-stained smears of female *Acanthocyclops vernalis*. (2) Uninucleate and multinucleate schizonts. (3) Multinucleated schizont undergoing synchronous nuclear division. (4–5) Rosette-shaped sporogonial plasmodia. (6) Group of non-membrane bound early stage sporoblasts immediately following cytoplasmic budding of the sporogonial plasmodium. (7) Fresh live spores (differential interference contrast).

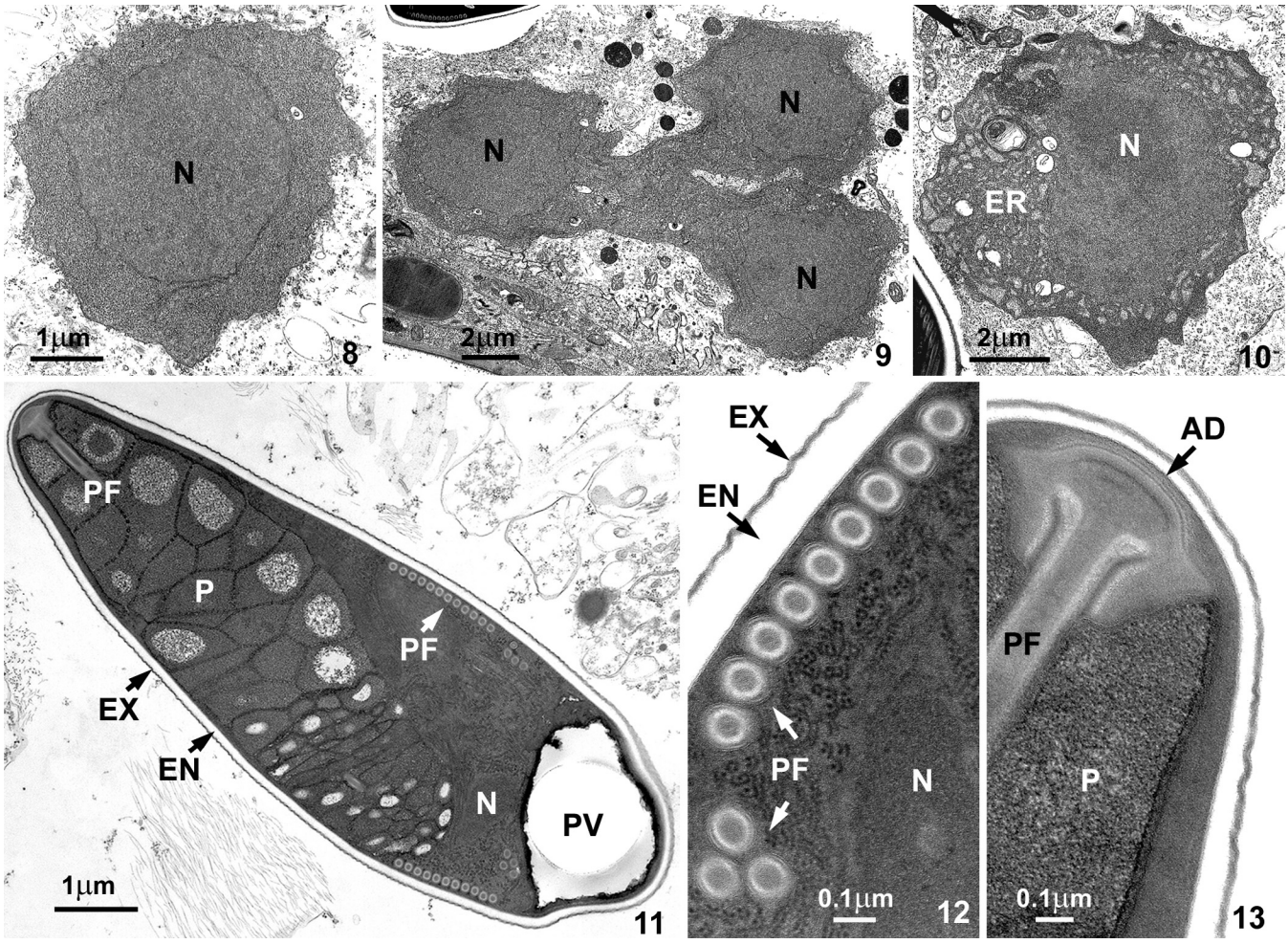

Fig. 8–13. Transmission electron micrographs of *Amblyospora khaliulini* development in female *Acanthocyclops vernalis.* (8) Uninucleate schizont. (9) Non membrane bound sporogonial plasmodium. (10) Early uninucleate sporoblasts with vacuolated cytoplasm and network of endoplasmic reticulum (ER). (11–13) Mature uninucleate spores. AD, anchoring disc; EN, endospore; EX, exospore; N, nucleus; P, polaroplast; PF, polar filament; PV posterior vacuole.

well developed and the polar sac was distinctly umbrella shaped (Fig. 23). The exospore was undulating and measured 0.25 μm, while the thinner electron lucent endospore measured 0.1 μm.

3.3. Development in Aedes communis: Horizontally acquired infections

Elucidation of horizontally acquired infections in *Ae. communis* was ascertained from direct examination of field collected individuals and corroborated through concurrent examination of third instar larvae that were exposed to mature spores from field collected *A. vernalis* copepods in the feeding trials. An infection rate of 32.8% (n = 64) was recorded in exposed mosquito larvae in the feeding trials which was significantly greater than the 11.3% (n = 71) recorded in the cohort of field-collected controls that were not exposed to copepod spores (X^2 = 8.0, P = .005).

Horizontally acquired infections were restricted to gonadal tissue in both male (testes) and female (ovaries) *Ae. communis* (Figs. 25, 26). The initial stages of development observed in third instar larvae were small (10 μm) uninucleate schizonts that were mostly fusiform with a nucleus at one pole (Fig. 27). No evidence of plasmogamy (cytoplasmic pairing) involving these stages was observed, but rather they appeared to undergo nuclear division (karyokinesis) (Fig. 28,29) to form diplokaryotic meronts. These transitional stages were distinguishable in ultrastructure by the possession of a highly irregular undulating plasmalemma and two intimately associated nuclei that were separated by a weakly defined nuclear envelope (Fig. 34). Synchronous nuclear division of diplokarya was further observed followed by cytoplasmic division which resulted in the formation of well-defined daughter diplokarya which were the predominant stage found in emerging adults (Fig. 30,31, and Fig. 35,36).

Further sporogonic development in adult males appeared aberrant despite the prolific development of infection in the testes, and was discerned during the early stages of sporogenesis. This was characterized by the formation of diplokaryotic sporonts with irregular undulating plasmalemmas and an abnormally extensive network of vacuoles within the cytoplasm (Fig. 37). Malformed residual spores void of any structural organelles (i.e. polar filament, polaroplast, posterior vacuole, endospore and exospore) were subsequently detected (Fig. 38).

Sporogenesis proceeded normally in adult females from progenitor diplokaryotic sporonts (Fig. 39) resulting in the formation of binucleate elliptical spores that measured 13.0 ± 0.8 μm × 5.0 ± 0.2 μm (fixed) (mean ± SD) (Fig. 32). Spores were thin walled (endospore = 0.1 μm, exospore = 0.04 μm), smooth and without ornamentation (Fig. 40,41). They possessed a large posterior vacuole and polaroplast composed of loosely arranged irregular membranes. The polar filament was isofilar with 9–11 irregularly arranged coils. The anchoring disc and polar sac were not well developed. Germinating spores with extruded polar filaments (Fig. 42) and empty spore "ghosts" (Fig. 33) were frequently observed within the ovaries of infected females.

Fig. 14. Heavily infected *Acanthocyclops vernalis* female showing distended ovaries (arrows) and mature spores.

Fig. 15. Fourth instar *Aedes communis* larva displaying patent late stage, vertically-acquired, *Amblyospora khaliulini* infection in fat body tissue within the thorax. Inset, fresh live spores (differential interference contrast) (X 10,000).

3.4. Natural epizootiology of infection

The prevalence rates of *A. khaliulini* infection in natural field populations of adult female *A. vernalis* and larval and adult *Ae. communis* ascertained from weekly examination of individuals collected from late March through mid-May over the course of four years are shown in Fig. 43 and summarized in Table 1.

Overall yearly infection rates in *A. vernalis* copepods were quite variable and ranged from a high of 57.1% in 2005 to a low of 15.0% in 2000 (yearly average = 29.5% ± 9.4 SE). With the exception of 1999, infection rates were typically highest in early spring (maximum of 92% in early April 2005), and thereafter declined through April and early May. This decline was accompanied by an over-all decrease in the size of copepod population based on sampling effort and their virtual disappearance in May coincident with drying of the pool.

The detection of horizontally acquired infections in 1 st through 4th instar *Ae. communis* larvae sampled weekly from late March through mid-May revealed prevalence rates of infection that were habitually low, ranging from 4.0% to 6.0% in each of the four years sampled (overall yearly average = 5.3% ± 0.5 SE). Weekly observations revealed consistently low infection rates throughout larval development with modest increases only among fourth instar larvae in late April and early May just prior to pupation and adult emergence. There was no significant association between horizontally acquired infections in *Ae. communis* larvae and infection rates observed concurrently in *A. vernalis* copepods (regression analysis where R = 0.45, P = .55, n = 4).

Prevalence rates of *A. khaliulini* infection in emerging adult *Ae. communis* were markedly higher than those recorded in larvae. There was no statistically significant difference in infection rates between the two sexes (paired t-test where t = 0.22, 6 df, P = .83). Infection rates in adult males ranged from 69.0% to 11.9% (yearly average = 39.2% ± 11.9 SE) and those in females ranged from 50.0% to 16.4% (yearly average = 36.1% ± 15.2 SE). As with larvae, there was no significant association between horizontally acquired infections in adult *Ae. communis* (combined assuming 1:1 sex ratio) and infection rates in *A. vernalis* copepods (regression analysis where R = 0.16, P = .84, n = 4).

Patent, vertically acquired infections detected in fourth instar *Ae. communis* larvae, determined just prior to pupation in early to mid-May of each year (Table 1), were consistently low ranging from 3.6% (n = 1218) in 2005 to 1.6% (n = 1398) in 2001 (yearly average = 2.7% ± 0.4 SE, n = 3166). As noted previously, these infections were restricted to male larvae.

4. Discussion

4.1. Interpretation of the life cycle

With this investigation we now detail the life cycle of *A. khaliulini* in both the mosquito and copepod hosts and corroborate the intermediary role of *A. vernalis* in support of previous molecular data confirming its identity (Vossbrinck et al., 2004). Consistent with other species of *Amblyospora,* we find that *A. khaliulini* exhibits polymorphic development, produces three morphologically and functionally distinct spores, and is both horizontally and vertically transmitted.

Development of *A. khaliulini* in *A. vernalis* is very similar to that described in other intermediate copepod hosts of *Amblyospora*. Infections are confined to females, occur within the ovaries preventing egg production and result in death of the host. Although copepods were not exposed to meiospores in experimental feeding trials, we infer that infections in *A. vernalis* arise from oral ingestion of meiospores produced in *Ae. communis* larvae which has been thoroughly documented in a number of other closely related species (Andreadis, 1985a, 1988a; Sweeney et al., 1985; Becnel, 1992; White et al., 1994; Becnel and Andreadis, 1998; Micieli et al., 1998, 2000a, 2000b). Parasite development in *A. vernalis* is entirely haplophasic and involves division by binary and multiple fission producing rosette-shaped sporogonial plasmodia and conical uninucleate spores with a characteristically large voluminous bipartite polaroplast and isofilar polar filament. Morphological differences with other described species of *Amblyospora* include the number of nuclei within the sporogonial plasmodia, the presence or absence of a sporogonial sporophorous vesicle, size of the spore, and number of coils of the polar filament which collectively appear to be species specific (Table 2).

We have shown that spores produced in *A. vernalis* are orally infectious to *Ae. communis* larvae. Both sexes are equally susceptible and infections are confined to gonadal tissue; testes in males and ovaries in females. We presume that spores germinate within the lumen of the

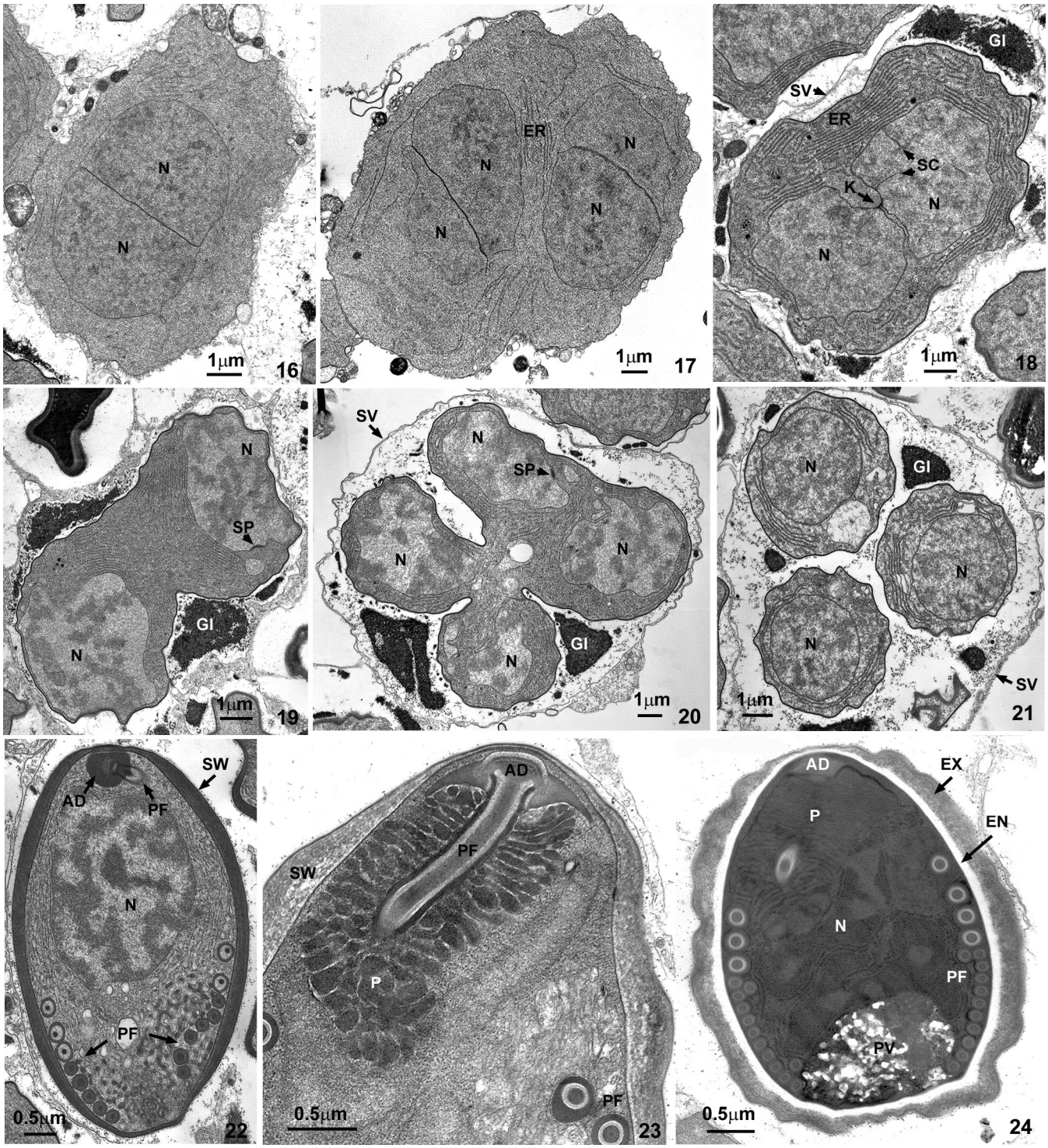

Fig. 16–24. Transmission electron micrographs of *Amblyospora khaliulini* development in fat body tissues of vertically-infected *Aedes communis* larvae. (16) Diplokaryotic meronts with closely paired nuclei (N). (17) Dividing diplokaryotic meront. (18) Diplokaryotic sporont during the initial stage of meiosis showing stacks of endoplasmic reticulum (ER), synaptonemal complexes (SC), crescent shaped kinetic center (K) separating each member of the diplokaryon and granular inclusions (GI) within the sporophorous vesicle (SV). (19) Binucleate sporont with spindle plaque (SP). (20) Multinucleated sporogonial plasmodium within the sporophorous vesicle (SV). (21) Early sporoblasts. (22, 23) Transitional sporoblasts showing differentiation of the anchoring disc (AD), polar filament (PF) and spore wall (SW). (24). Mature uninucleate spore. EN, endospore; EX, exospore; PV posterior vacuole.

midgut, but unlike other species of *Amblyospora* spp. (Andreadis, 1985b, 1988b) and the closely related *Edhazardia aedis* (Becnel et al., 1989; Johnson et al. 1997), *A. khaliulini* does not appear to initially infect the epithelial cells of the midgut or gastric caeca, but rather directly invades host gonadal tissue where all subsequent development occurs. This apparent bypass of the midgut epithelium has similarly been observed in *Hyalinocysta chapmani,* which directly infects larval fat body tissue in *Culiseta melanura* (Andreadis and Vossbrinck, 2002). Our failure to observe any early auto infective spores that function in dispersal of infection from the gastric caeca to other tissues as reported in *E. aedis* (Johnson et al., 1997), supports this conclusion.

The earliest stages of development observed in larvae following ingestion of spores from *A. vernalis* were small fusiform shaped uninucleate schizonts, consistent with the uninucleate nature of the

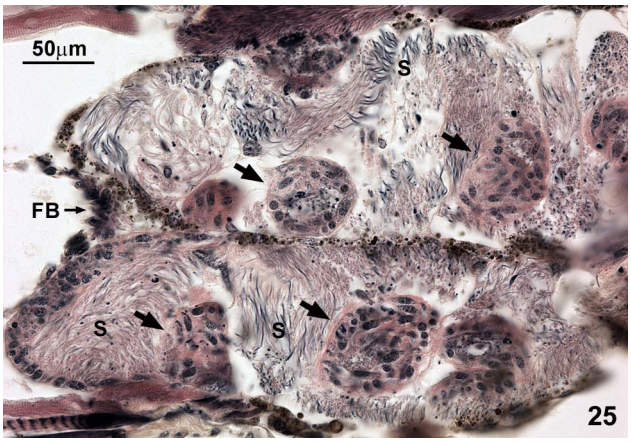

Fig. 25. Sagittal histological section through the abdomen of an adult male *Aedes communis* showing *Amblyospora khaliulini* infection (arrows) within the testes. FB, uninfected fat body; S, filamentous spermatozoa.

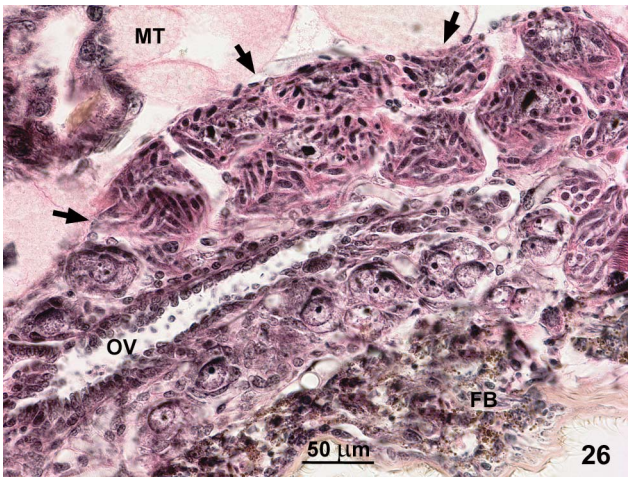

Fig. 26. Sagittal histological section through the abdomen of an adult female *Aedes communis* showing *Amblyospora khaliulini* infection (arrows) within the ovaries (OV). FB, uninfected fat body; MT, Malpighian tubules.

infectious spore. This was followed by the detection of transitional schizonts with diplokaryotic nuclei. The manner in which the diplokaryotic arrangement is achieved appears to be by karyokinesis (mitotic nuclear division), as we could find no evidence that early fusiform schizonts undergo plasmogamy (cytoplasmic fusion) and nuclear association, as has been documented in other species of *Amblyospora* (*A. californica*, *A. connecticus*, *A. salinaria*, *A. stimuli*) (Andreadis, 1985b, 1988b; Becnel, 1992; Becnel and Andreadis, 1998). However, this phenomenon is not unique, as a nearly identical method of karyokinesis of uninucleate schizonts has been similarly noted in *H. chapmani* (Andreadis and Vossbrinck, 2002) and *Takaokaspora nipponicus* (host = *Aedes japonicus*) (Andreadis et al., 2013).

Diplokaryotic meronts next exhibit a proliferative phase of development during which they divide repeatedly by binary fission to produce large numbers of diplokaryotic daughter cells. This asexual phase of multiplication occurs in both host sexes but sporogenesis does not progress normally in adult male hosts and functional binucleate spores are formed in adult females only. This is a common manifestation of infection seen in almost all other species of *Amblyospora* (*A. albifasciati, A. californica, A. camposi, A. connecticus, A. ferocis, A. salinaria)* that have been examined thus far (Becnel, 1992; Becnel and Andreadis, 1998; Micieli et al., 2000a, 2000b; Andreadis and Vossbrinck, 2002). The two noted exceptions include *Amblyospora indicola* from *Culex sitiens* (Sweeney et al., 1990) and *Amblyospora weiseri* from *Aedes cantans* (Lukes and Vavra, 1990), which reportedly form binucleate spores in a proportion of both male and female mosquitoes.

The functional significance of these infections in adult male *Ae. communis* as well as all other species of *Amblyospora* is unresolved, as no evidence of paternal mediated vertical transmission of these mosquito-parasitic microsporidia has ever been demonstrated. We can only surmise that infections in adult males represent a vestigial remnant in the life cycle. This view is consistent with recently completed comparative phylogenetic analyses demonstrating a high degree of host-parasite co-speciation among the entire group of mosquito-parasitic species (Andreadis et al., 2012), and the hypothesis that these microsporidia evolved from parasites of crustaceans and proceeded to adapt their life cycles to accommodate specific host ecological conditions (Vossbrinck et al., 2004; Vossbrinck and Debrunner-Vossbrinck, 2005).

Binucleate spores formed in adult female mosquitoes function in vertical transmission of the microsporidium via infection of the ovaries and eggs. Unlike most other species of *Amblyospora* (*A. campelli* from *Culiseta campbelli*, *A. connecticus* from *Ae. cantator*, *A. salinaria* from *Cx. salinarius*, *A. stimuli* from *Ae. stimulans*) (Andreadis and Hall, 1979; Andreadis, 1983a, 1985b; Dickson and Barr, 1990), a blood meal is not required for the initiation of *A. khaliulini* sporogenesis in female *Ae. communis* and spore germination appears to proceed spontaneously. Similar findings with spore formation in the absence of a blood meal have been reported in *A. dyxenoides* from *Cx. anulirostris* (Sweeney et al., 1988) and *A. weiseri* from *Ae. cantans* (Lukes and Vavra, 1990). Aside from the size of the spore and number of coils in the polar filament, binucleate spores formed in adult females were morphologically similar to the only other three species (*A. connecticus, A. ferocis*, *A. salinaria*) that have been examined thus far at the ultrastructural level (i.e. isofilar irregularly arranged polar filament, thin unadorned exospore and endospore, large loosely arranged membranous polaroplast and large posterior vacuole) (Andreadis 1983a, 1988b; Lukes and Vavra, 1990; Micieli et al., 2003).

Because *Ae. communis* is a univoltine mosquito which will not mate in the laboratory, it was not possible to unequivocally demonstrate that binucleate spores formed in adult females were responsible for vertical transmission of infection to larval progeny and that the resulting infection leading to the production of meiospores was acquired via this route. However, this aspect of the life cycle has been well documented and is universally recognized (Andreadis, 2007 and references therein), so we find no contradictions in inferring this function.

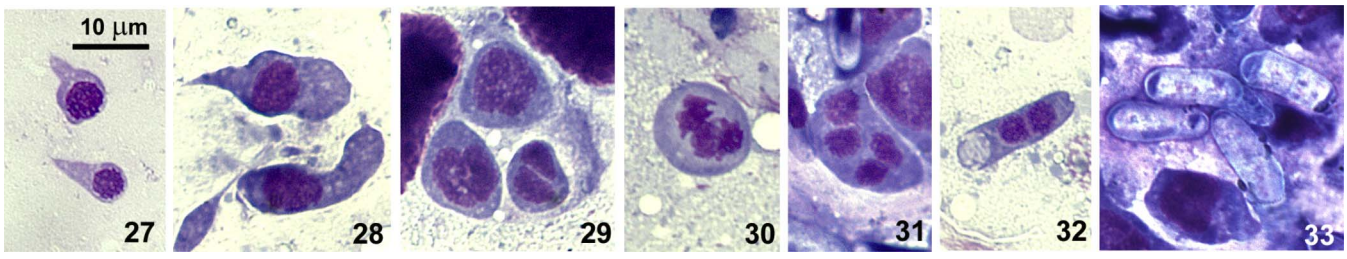

Fig. 27–33. Developmental stages of *Amblyospora khaliulini* as observed in Giemsa-stained smears of horizontally (orally) infected *Aedes communis*. (27) Early stage fusiform uninucleate schizont from 3rd instar larva. (28–29) Schizonts exhibiting karyokinesis. (30, 31) Dividing diplokaryotic meronts. (32) Binucleate spore. (33) Extruded spore.

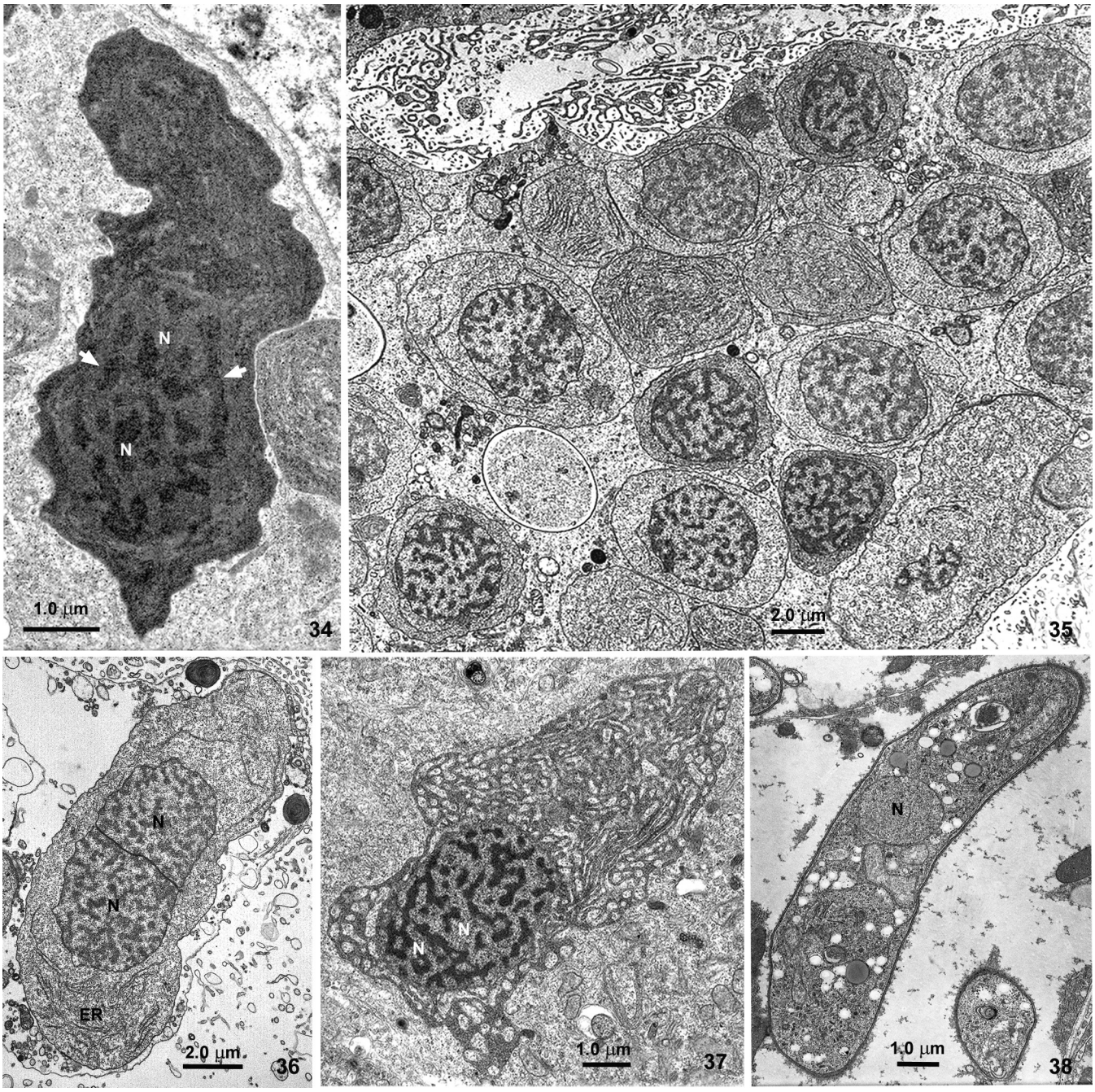

Fig. 34–38. Transmission electron micrographs of *Amblyospora khaliulini* development in the testes of horizontally-infected adult male *Aedes communis*. (34). Transitional schizont with undulating plasmalemma and associated nuclei (N) separated by a weakly defined nuclear envelope (arrows). (35). Cross section through a group of diplokaryotic meronts. (36). Diplokaryotic meronts with arrays of endoplasmic reticulum (ER). (37). Diplokaryotic sporont with undulating plasmalemma and extensive network of vacuoles within the cytoplasm. (38). Aberrant spore.

Development of vertically acquired *A. khaliulini* infections in larval *Ae. communis* hosts occurs within fat body tissue, leads to the production of meiospores and appears to occur in male hosts only. This restriction of patent fat body infections to males is not unique and has been similarly observed in a number of other species (*A. bolinasae, A. californica, A. gigantea, A. salinaria*, *A. stictici, A. stimuli*) (Kellen et al., 1965; Chapman et al., 1966; Andreadis and Hall, 1979; Andreadis, 1985b). The initial stages of development observed in male larvae are diplokaryotic meronts, consistent with the binucleate nature of the spores found in the parental females. Development is initially characterized by merogonial multiplication of diplokarya by synchronous binary division producing additional diplokarya which infect other fat body cells and thus spread infection. The cessation of merogony and the onset of sporogony are characterized by the simultaneous secretion of a sporophorous vesicle and meiotic division of the diplokaryon. This ultimately results in the formation of octonucleate sporonts that then undergo cytokinesis and sporogenesis to form eight meiospores enclosed within a sporophorous vesicle. The ultrastructural features associated with the meiotic sequence and sporogonial phase of development in *A. khaliulini* are virtually identical to those reported in other *Amblyospora* spp. (Andreadis and Hall, 1979; Andreadis, 1983a; Vavra et al., 1984; Sweeney et al., 1988; Becnel and Sweeney, 1990; Micieli et al., 2000a). Infected larvae die during the 4th stadium and with the exception of the size of the meiospores and number of coils in the polar filament, which are uniquely diagnostic, the ultrastructural spore morphology of *A. khaliulini* is very similar to that described for other

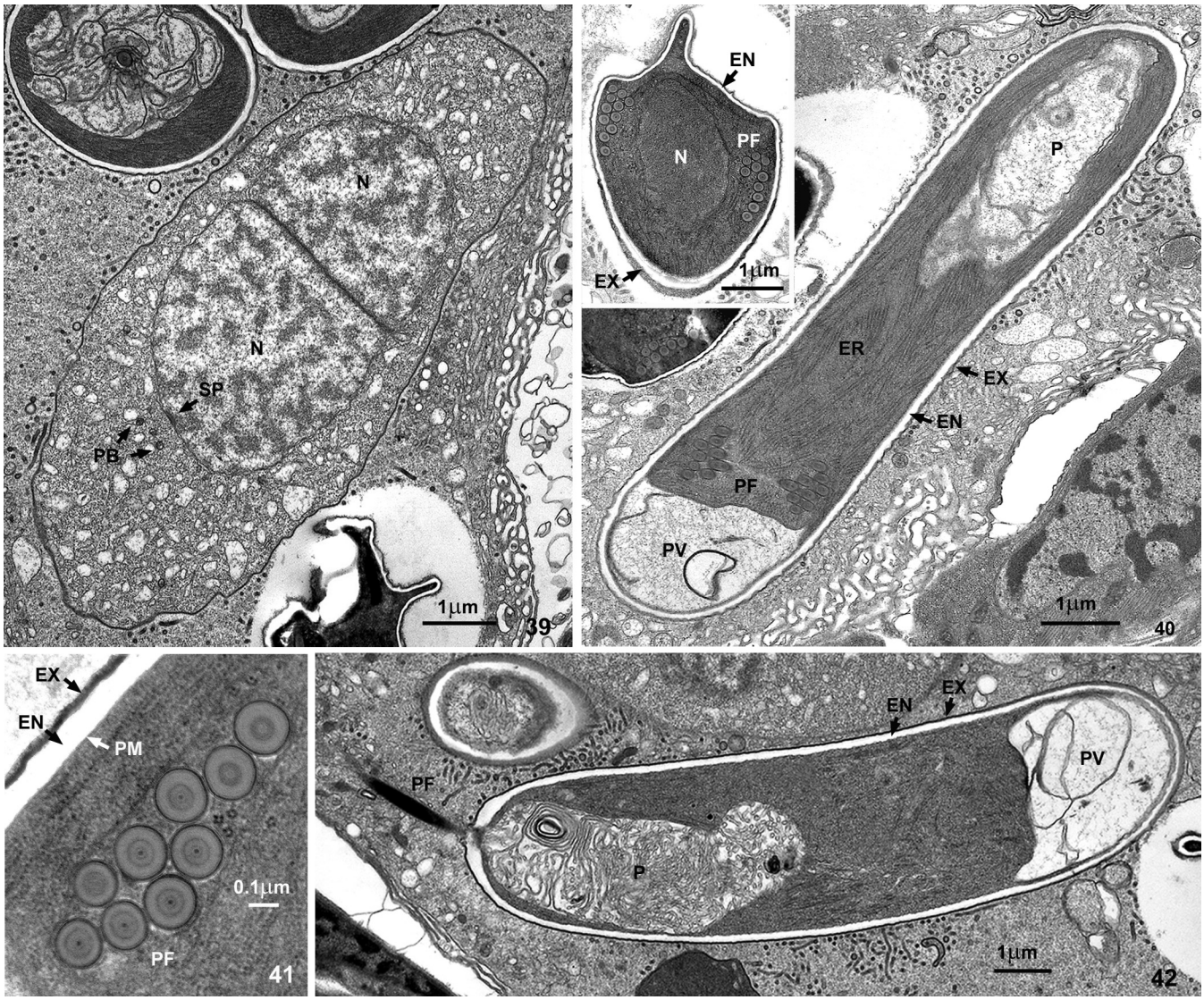

Fig. 39–42. Transmission electron micrographs of *Amblyospora khaliulini* development in the ovaries of horizontally-infected adult female *Aedes communis.* (39). Diplokaryotic sporont with spindle plaque (SP) and polar vesicles (PR). (40, 41). Sagittal and cross (inset) sections of mature binucleate spores. (42). Spore with extruded polar filament (PF). EN, endospore; EX, exospore; N, nucleus; P, polaroplast; PM, plasmalemma; PV posterior vacuole.

Amblyospora species (Andreadis, 1994; Andreadis et al., 2012).

We were not able to ascertain whether or not a proportion of the female progeny develop vertically acquired benign infections that eventually lead to the formation of binucleate spores as occurs in the oenocytes of at least four other species of *Amblyospora* (*A. californica, A. connecticus, A. salinaria,* and *A. stimuli*) (Andreadis and Hall, 1979; Andreadis, 1983a, 1985b; Becnel, 1992). This is because this developmental sequence essentially mimics that which arises horizontally via oral ingestion of spores from the intermediate copepod host, and cannot be reliably differentiated in the absence of obtaining eggs from infected *Ae. communis* females and rearing progeny in the laboratory, which was not possible with this univoltine mosquito.

4.2. Epizootiology

Our observations on the natural prevalence of *A. khaliulini* infection in field populations of *Ae. communis* revealed consistently low rates of patent vertically acquired fat body infections in male larvae that ranged from 1.6% to 3.6% (mean = 2.7%) despite the comparatively high rates of infection in parental females. Since this is the only phase of the life cycle that is overtly detrimental to the host, we conclude that *A. khaliulini* has minimal impact on *Ae. communis* populations at this location. This contrasts somewhat with the 3% to 11% prevalence rates of *A. khaliulini* infection reported by Welch (1960) in larval populations of *Ae. communis* inhabiting forest pools in Manitoba, Canada, but is comparable to the 1.3% to 5.9% (mean = 3.6%) prevalence of *A. stimuli* infection in natural populations of *Ae. stimulans*, an ecologically similar forest dwelling, vernal pool inhabiting, univoltine mosquito (Andreadis, 1999). Although omnipresent, most species or isolates of *Amblyospora* infecting northern univoltine mosquitoes typically infect less than 1% of the larval population (Anderson, 1968; Chapman, 1974), and their ability to induce high infection rates with these lethal vertically induced infections is apparently quite rare if nonexistent. Bona fide epizootics of *Amblyospora* involving natural larval mosquito populations have been reported but only among multivoltine mosquito hosts. This results from the synchronized hatch of vertically infected eggs in the autumn of the year with prevalence rates of infection approaching 100% (Andreadis, 1983b, 1990, 1993; Micieli et al., 2003).

The detection of moderate to high rates of infection in adult female *A. vernalis* copepods in the early spring coincident with ice melt in the vernal pool, leads us to conclude that *A. khaliulini* most likely overwinters in the intermediate copepod host as well as in dormant eggs of

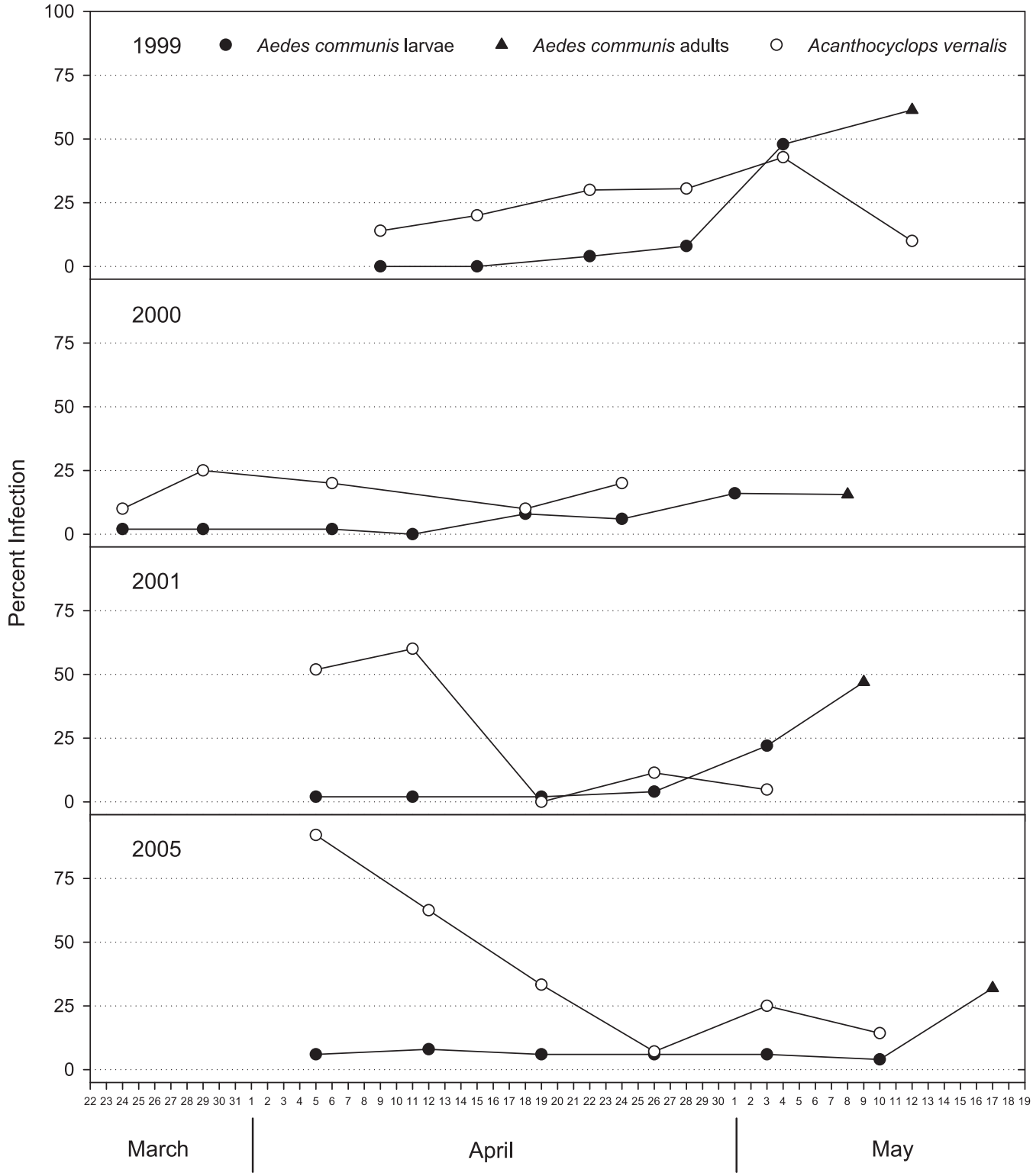

Fig. 43. Prevalence of *Amblyospora khaliulini* infection in adult female *Acanthocyclops vernalis* copepods and larval and emerging adult populations of *Aedes communis* (horizontally acquired) within a vernal pool habitat in Barkhamsted, CT 1999–2001 and 2005.

the mosquito host, *Ae. communis*. This view is consistent with the life history of *A. vernalis* which is reported to have one or two generations a year in north-temperate regions and overwinter as a diapausing 4th or 5th stage copepodid (Andrews, 1953; Selgeby, 1975). This presumes that developing copepodids acquire infection via ingestion of meiospores that are released into the pool with the death of infected *Ae. communis* larvae later in the spring. This same scenario with overwintering in *A. vernalis* copepods has been documented for *A. connecticus*, a parasite of *Ae. cantator* (Andreadis, 1990). We attribute the precipitous decline in the prevalence of *A. khaliulini* infection in *A. vernalis* populations observed during the course of the spring to parasite induced mortality, as overall copepod abundance similarly declined over the same period.

The comparatively low prevalence rates of horizontally acquired

Table 1
Yearly prevalence rates of *Amblyospora khaliulini* infections in field populations of *Acanthocyclops vernalis* copepods and *Aedes communis* mosquitoes inhabiting a vernal pool site in Barkhamsted, Connecticut.

Year	*Acanthocyclops vernalis*	*Aedes communis*			
		Vertically acquired infection in larvae	Horizontally acquired infection		
			Larvae	Adults	
				Male	Female
1999	22.1 (n = 240)	2.5 (n = 200)	4.0 (n = 200)	69.0 (n = 100)	45.9 (n = 111)
2000	15.0 (n = 153)	3.1 (n = 350)	4.9 (n = 350)	11.9 (n = 59)	16.4 (n = 55)
2001	23.9 (n = 172)	1.6 (n = 1398)	6.4 (n = 250)	44.0 (n = 50)	50.0 (n = 50)
2005	57.1 (n = 105)	3.6 (n = 1218)	6.0 (n = 300)	32.0 (n = 50)	32.0 (n = 50)

infections of *A. khaliulini* in *Ae. communis* larvae and substantially higher prevalence rates observed in emerging adults are enigmatic. The low levels of infection observed in early instar *Ae. communis* larvae suggest to us that either these early instars are not susceptible to infection or alternatively do not feed on, or encounter spores that are released into the pool with the death of infected copepods. It was not possible to directly measure the inoculum of infectious copepod spores in the pool that were available for ingestion by *Ae. communis* larvae during the course of their development. However, based on the detection and abundance of spore laden copepods early in the season, and their apparent mortality over the course of several weeks, we do not feel "spore load" was a limiting factor unless their distribution in the pool was simply too sparse. The alternative explanation, that early instar larvae are simply not susceptible to infection could be a function of the site of apparent infection, the gonads. The testes in males are recognizable as early as the 2nd instar and are quite conspicuous in the 3rd (Christophers, 1960). However, it is not until the 4th instar that the greatest increase in length takes place and each gonad becomes invested by fat cells and layers of spermatogonia and spermatocytes (Horsfall and Ronquillo, 1970). Similarly, the ovaries are present in the 3rd instar, but it is during the 4th instar that they become elongate structures with early follicle formation (Christophers, 1960). The suggestion that late instar larvae are more susceptibility to infection is supported anecdotally by our repeated detection of increases in the prevalence of infection in 4th instar larvae just prior to pupation. Clearly, additional laboratory transmission trials with early instar larvae are needed to resolve this issue as we only evaluated the susceptibility of 3rd instars. Lastly, we cannot completely discount the possibility that the levels of microsporidian infection in early instar larvae were simply too low to detect using light microscopy. However, detection of as few as a handful of vegetative stages has been reliably detected using the methods of microscopic examination of Giemsa-stained smears of early instar mosquito larvae (Andreadis, 1985b, 1990, 1999; Andreadis and Vossbrinck, 2002).

5. Taxonomic summary and amended description

5.1. *Amblyospora khaliulini*

Hazard and Oldacre, 1975

5.1.1. *Type definitive host*
Aedes communis (DeGeer) (Diptera: Culicidae). GenBank/EMBL database Accession No. AY988425.

5.1.2. *Type intermediate host*
Acanthocyclops vernalis (Fisher) (Copepoda, Cyclopidae).

5.1.3. *Type locality*
South Deerfield, Massachusetts, USA (holotype).

5.1.4. *Additional localities*
Barkhamsted, Connecticut, USA (paratypes); Sagwon, Alaska, USA; Churchill, Manitoba, Canada; Czech Republic; Germany; Russia.

5.1.5. *Transmission*
Horizontal to *Ae. communis* larvae (both sexes) via oral ingestion of uninucleate spores from *A. vernalis*. Horizontal to female *A. vernalis* via oral ingestion of meiospores from *Ae. communis* larvae. Vertical (transovarial) from adult female *Ae. communis* to male larval progeny via ovarian infect with binucleate spores

5.1.6. *Site of infection*
Ae. communis, fat body tissue of vertically-infected larvae; gonadal tissue in horizontally infected males (testes) and females (ovaries). *A. vernalis*, ovaries of adult females.

5.1.7. *Development*
Polymorphic producing three spore types. (1) Development in horizontally infected *Ae. communis* larvae occurs through oral ingestion of uninucleate spores from *A. vernalis* with development restricted to gonadal tissue. Uninucleate schizonts undergo karyokinesis forming diplokaryotic meronts which divide repeatedly by synchronous binary fission. Sporogonic development aberrant in adult males. Sporogenesis in adult females results in non-membrane bound binucleate spores that function in vertical transmission of infection to larval progeny. (2) Development in vertically infected *Ae. communis* restricted to fat body tissue of males. Initial merogonial multiplication of diplokarya by synchronous binary division. Sporogony characterized by simultaneous secretion of a sporophorous vesicle and meiotic division forming octonucleate sporonts that undergo cytokinesis forming eight haploid

Table 2
Comparison of salient diagnostic features of mature spores of *Amblyospora* species identified from intermediate copepod hosts.

Species	Intermediate copepod host	Sporophorous vesicle	No. nuclei sporogonial plasmodium	Spore size	No. coils polar filament	Reference
A. albifasciati	*Mesocyclops annulatus*	Yes	4–8	10.4 × 4.8 μm	9–10	Micieli et al. (2000b)
A. californica	*Macrocyclops albidus*	Yes	4	13.2 × 3.8 μm	10–11	Becnel (1992)
A. camposi	*Paracyclops fimbriatus fimbriatus*	Yes	4–8	10.7 × 3.8 μm	7–8	Micieli et al. (2000a)
A. connecticus	*Acanthocyclops vernalis*	Yes	12	9.0 × 5.5 μm	11–12	Andreadis, 1985a, 1985b
A. dolosi	*Metacyclops mendocinus*	No	4–6	14.3 × 3.8 μm	12–13	Micieli et al. (1998)
A. dyxenoides	*Mesocyclops albicans*	No	4	12.5 × 4.6 μm	11–12	Sweeney et al. (1985)
A. khaliulini	***Acanthocyclops vernalis***	**No**	**8**	**11.3 × 4.4 μm**	**12–15**	**Present study**
A. opacita	*Paracyclops fimbriatus chiltoni*	No	2–4	15.2 × 4.0 μm	15–17	White et al. (1994)
A. salinaria	*Macrocyclops albidus*	Yes	4	13.2 × 3.8 μm	12–14	Becnel and Andreadis (1998)

meiospores enclosed in a persistent sporophorous vesicle. (3) Development in horizontally infected *A. venalis* confined to ovarian tissue in adult females entirely haplophasic. Involves division of uninucleate schizonts by binary and multiple fission producing rosette-shaped plasmodia with up to eight nuclei followed by cytokinesis forming non-membrane bound uninucleate spores.

5.1.8. Spore morphology. Meiospore

Uninucleate, broadly ovoid, measuring 7.0 ± 0.1 × 4.7 ± 0.1 µm (live). Polar filament anisofilar with 3–4 broad proximal and 11–12 narrow distal coils arranged in a single row. Polaroplast bilaminar with more closely packed anterior lamellae. Anchoring disc well developed, polar sac umbrella shaped and large posterior vacuole containing a mesh-like matrix. Exospore undulating, 0.25 µm thick and endospore measuring 0.1 µm. *Binucleate spore:* Binucleate, elliptical, measuring 13.0 ± 0.8 µm × 5.0 ± 0.2 µm (fixed). Polar filament isofilar with 9–11 irregularly arranged coils. Polaroplast with loosely arranged irregular membranes. Anchoring disc and polar sac rudimentary but with large posterior vacuole. Spore wall thin (endospore = 0.1 µm, exospore = 0.04 µm), smooth and without ornamentation. *Copepod spore:* Uninucleate, conical measuring 11.3 ± 0.4 × 4.4 ± 0.1 µm (live). Polar filament isofilar with 12–15 coils arranged in a single row but with notable clustering of the last three most posterior turns. Polaroplast voluminous, and bipartite consisting of large irregularly spaced vesicular chambers in the anterior end and more tightly compressed lamellar elements in the posterior end. Anchoring disc of the polar sac well developed, large posterior vacuole. Exospore slightly undulating, without ornamentation measuring 20 nm, endospore measuring approximately 100 nm.

5.1.9. Bionomics

Ecological habit a leaf-lined vernal pool within an evergreen forest dominated by eastern hemlock (*Tsuga canadensis*) and eastern white pine (*Pinus strobus*). Prevalence of vertically acquired fat body infections in male *Ae. communis* larvae 1.6% to 3.6% (mean = 2.7%). Yearly infection rates in *A. vernalis* copepods 57.1% to 15.0% (mean = 29.5%). Prevalence rates of horizontally acquired infections in emerging adult *Ae. communis* 69.0% to 11.9% (mean = 39.2%) in males and 50.0% to 16.4% (mean = 36.1%) in females.

5.1.10. Type material

Four voucher slides have been deposited with the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, DC (USNM Nos. 1437652, 1437653, 1437654, 1437655). Additional voucher slides consisting of: (1) Giemsa-stained smears and paraffin embedded histological sections of infected tissues from larval and adult *Ae. communis* and female *A. vernalis,* (2) plastic embedded tissues from adult *Ae. communis* and *A. vernalis* used in the ultrastructural investigations, and (3) frozen DNAs of the *A. khaliulini* from each host are in the collection of Theodore G. Andreadis, Center for Vector Biology & Zoonotic Diseases, The Connecticut Agricultural Experiment Station, New Haven CT.

5.1.11. Gene sequences

The SSU rDNA sequences of the microsporidium, *A. khaliulini* have been deposited in the GenBank/EMBL database Accession Nos. AY090045 (*Ae. communis*) and AY090046 and AY090047 (*A. vernalis*)

Acknowledgements

This study was supported in part by US Department of Agriculture grant CONH00768 issued to TGA.

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