J. Eukaryot. Microbiol., 49(4), 2002 pp. 350–364

Life Cycle, Ultrastructure and Molecular Phylogeny of *Hyalinocysta chapmani* (Microsporidia: Thelohaniidae), a parasite of *Culiseta melanura* (Diptera: Culicidae) and *Orthocyclops modestus* (Copepoda: Cyclopidae)

THEODORE G. ANDREADIS and CHARLES R. VOSSBRINCK

The Connecticut Agricultural Experiment Station, 123 Huntington Street, PO Box 1106, New Haven, Connecticut 06504, USA

ABSTRACT. The complete life cycle of the microsporidium *Hyalinocysta chapmani* is described from the primary mosquito host *Culiseta melanura* and the intermediate copepod host *Orthocyclops modestus*. Infections are initiated in larval *C. melanura* following the oral ingestion of uninucleate spores from infected copepods. Spores germinate within the lumen of the midgut and directly invade fat body tissue where all development occurs. Uninucleated schizonts undergo binary division (schizogony) followed by karyokinesis (nuclear division) to form diplokaryotic meronts. Merogony is by synchronous binary division. The onset of sporogony is characterized by the simultaneous secretion of a sporophorous vesicle and meiotic division of the diplokaryon resulting in the formation of eight ovoid meiospores enclosed within a sporophorous vesicle. Most infected larvae die during the fourth stadium and there is no evidence of a developmental sequence leading to vertical transmission. *Hyalinocysta chapmani* is horizontally transmitted to *O. modestus* via oral ingestion of meiospores. Infections become established within ovarian tissue of females and all parasite development is haplophasic. Uninucleate schizonts divide by binary division during an initial schizogonic cycle. Newly formed uninucleate cells produce a thin sporophorous vesicle and undergo repeated nuclear division during sporogony to produce a rosette-shaped, multinucleated sporogonial plasmodium with up to 18 nuclei. This is followed by cytoplasmic cleavage, sporogenesis, and disintegration of the sporophorous vesicle to form membrane-free uninucleate spores. Infected females eventually die and there is no egg development. The small subunit rDNA sequence of *H. chapmani* isolated from meiospores from *C. melanura* was identical to the small subunit rDNA sequence obtained from spores from *O. modestus*, corroborating the laboratory transmission studies and confirming the intermediary role of *O modestus* in the life cycle. Phylogenetic analysis was conducted with closely related microsporidia from mosquitoes. *Hyalinocysta chapmani* did not cluster within described *Amblyospora* species and can be considered a sister group, warranting separate genus status.

Key Words. Copepod, development, horizontal transmission, intermediate host, Microsporidia, mosquito, small subunit ribosomal DNA sequence, taxonomy.

Hyalinocysta chapmani Hazard and Oldacre, 1975 is a little known microsporidian parasite of the mosquito *Culiseta melanura* Coquillett (Diptera: Culicidae). The microsporidium was originally discovered by Dr. Harold Chapman, who observed patent infections in a few larval mosquitoes collected in southern Louisiana, USA in 1971. *Hyalinocysta chapmani* was formally described by Hazard and Oldacre (1975) who created a new genus with *H. chapmani* as the type species by monotypy. Their description was based on the absence of secretory products in the sporophorous vesicle, and the shape and ultrastructural morphology of the *Amblyospora*-like ''octospore'' (meiospore) found in larval fat body tissue. No details on the life cycle, pathology or mode(s) of transmission were given at that time. One additional species of *Hyalinocysta* has since been described from Scania, Sweden: *Hyalinocysta explitoria* Larsson, 1983, a parasite of the blackfly, *Simulium ornatum* Meigen (Diptera: Simuliidae) (originally identified as *Odagmia ornata* (Meigen)) (Larsson 1983, 1989).

While conducting a field survey for microbial parasites of mosquitoes in Connecticut, USA, *H. chapmani* was rediscovered in a larval population of *C. melanura* inhabiting a freshwater swamp. The present report provides a complete characterization of the life cycle, developmental pathways, and histopathology of *H. chapmani* in all stages of *C. melanura* by light and electron microscopy. We further describe the mechanisms of horizontal transmission and document the intermediary role of *Orthocyclops modestus* (Herrick) (Copepoda: Cyclopidae) in the life cycle. We also provide small subunit rDNA sequences for *H. chapmani* from both its mosquito and copepod host and determine the phylogenetic position of *Hyalinocysta* among related microsporidia.

MATERIALS AND METHODS

Life cycle studies. The life cycle studies were conducted over a three-year period (1995–1997) with naturally infected specimens obtained from a subterranean habitat in a fresh water swamp (sphagnum bog) at Mohawk State Forest in Cornwall, (Litchfield County), CT (41° 48′ 45′pr N, 73° 17′ 41′pr W). The developmental pathways and life history of *H. chapmani* were constructed from chronological examination of all stages (i.e. egg, larva, pupa, adult male, blood- and non-blood-fed adult females) of the definitive mosquito host, *C. melanura*, and the intermediate copepod host, *O. modestus* (i.e. nauplius, copepodid, adult male, gravid and non-gravid adult female). Concurrent investigations with laboratory-infected specimens were additionally undertaken to corroborate observations on field-collected material. The procedures for infecting hosts are described in the transmission studies section below.

General characterization of microsporidian development was made from microscopic (1,000×) examination of Giemsa-stained smears (10% solution, pH 6.8) of infected tissues dissected from live hosts. The prevalence of individual stages of *H. chapmani* was quantified in each respective host stage to help ascertain the correct sequence of events in the life cycle. Tissue specificity was determined from histological examination of paraffin-embedded whole specimens stained with iron hematoxylin and eosin Y and unstained plastic-embedded specimens using phase contrast microscopy (Andreadis 1988a, b). Measurements of mature spores were calculated from photomicrographs of live spores (n = 50) from each host (mosquito and copepod) as observed in whole wet-mount preparations with phase contrast microscopy (1000×).

Complete characterization of all stages in the life cycle was made at the ultrastructural level. Whole copepods and infected tissues of *C. melanura* larvae (1 mm^3) were fixed in a 2.5% (v/v) glutaraldehyde solution containing 0.1% (w/v) $CaCl_2$ and 1% (w/v) sucrose in 100 mM Na cacodylate (pH 7.3), postfixed in aqueous 1% (w/v) OsO_4, stained en bloc in 2% (w/v in 70% ETOH) uranyl acetate, dehydrated through an ascending ethanol and acetone series and embedded in a LX-112/Araldite (Ladd Research Industries, Williston, VT) mixture. Thin serial

The small subunit rDNA sequence of *Hyalinocysta chapmani* has been deposited in the GenBank database under accession numbers AF483837 (*Culiseta melanura*) and AF483838 (*Orthocyclops modestus*).

Corresponding Author: T. Andreadis–Telephone number: (203) 974-8510; FAX number: (203) 974-8502; E-mail: theodore.andreadis@po.state.ct.us

sections showing gold (for spores) and silver (for vegetative stages) interference color (60–90 nm) were cut with a diamond knife and post-stained with 5% (w/v) uranyl acetate in 50% (v/v) methanol followed by Reynold's lead citrate (Andreadis 1983). These were examined in a Zeiss EM 10C electron microscope at an accelerating voltage of 80 kV.

Transmission studies. Experimental horizontal transmission studies were conducted with both hosts in the laboratory. Feeding trials were undertaken at various times of the year depending on the availability of copepods and infected hosts. Fresh mature spores harvested from naturally infected *C. melanura* larvae and *O. modestus* copepods were used as inocula in all trials. The *C. melanura* larvae used in the mosquito exposure trials were obtained from a disease-free laboratory colony; the *O. modestus* copepods were field-collected. Gravid female copepods (i.e. bearing egg sacs) were used in the later trials, as no natural infections were found in this stage. All bioassays were conducted at 25 °C, under a 16:8 LD photoperiod, in 100 × 80-mm culture dishes containing 100 ml of distilled water.

In the mosquito exposure trials, 50 second instar *C. melanura* larvae were placed in a culture dish along with two macerated, infected *O. modestus* copepods each containing approximately 1×10^4 mature spores. A small amount of an aqueous suspension of liver powder and Brewer's yeast was provided for food.

In the copepod exposure trials, 25 female *O. modestus* copepods were similarly placed in the culture dishes and exposed to meiospores from 2 infected mosquito larvae (approximately 1×10^5 spores/larva). Copepods were supplied with a small amount of food consisting of bacteria, mixed protozoa, and Brewer's yeast. Control dishes contained an equal number of mosquito larvae or copepods and food, but were not exposed to microsporidia spores. Individual copepods and mosquitoes were examined for infection at 7, 14 and 21 days post-exposure. On each occasion, 5 copepods or 10 mosquito larvae were removed from each dish, smeared on slides, stained with Giemsa, and examined microscopically (1000×) as described previously. Pupating mosquitoes were allowed to emerge and were examined for infection as adults. The type and prevalence of infection were recorded in each host. A limited number of bioassays were also conducted in which *C. melanura* larvae and *O. modestus* copepods were similarly exposed to fresh live spores obtained from the same host species.

The potential for maternal-mediated vertical transmission of *H. chapmani* in *C. melanura* was further assessed by examining progeny reared from adult females that had been previously exposed as larvae to spores from *O. modestus*. These females were held in 30.5-cm^3 screened cages with an equal number of healthy males (from colony) for 24 h at 25 °C and were then allowed to blood feed on restrained button quail, *Cotumix chinensis*. Blood-fed females were individually transferred to a 15-cm^3 screened cage for oviposition. Cages were checked daily for eggs, and following oviposition or death, each female was examined for infection (Giemsa-stained smear). Sibling larvae were reared collectively in 100 × 80 mm culture dishes and were similarly examined for infection during the second and third stadium.

Molecular phylogeny. Nucleotide sequences were obtained from mature spores of *H. chapmani* that were procured from naturally-infected fourth instar *C. melanura* and adult female *O. modestus*. Methods were similar to those previously published (Vossbrinck, Andreadis, and Debrunner-Vossbrinck 1998). Approximately 10^5 spores were isolated from 1 mosquito larva or 2 copepods. Single mosquito larvae or copepods were homogenized in TAE buffer and filtered through a 50-μm nylon mesh. The filtrate was spun briefly and the supernatant was then removed. The pellet was re-suspended in 150 μl of TAE buffer and placed in a 0.5 ml microcentrifuge tube. Ten microliters of liquid were removed and examined under phase contrast microscopy (100×) to confirm the presence of viable spores, which typically appear highly refractive. One-hundred-fifty milligrams of glass beads were then added and the tube was shaken in a Mini-Beadbeater (Biospec Products Bartlesville, OK) for 50 s and placed immediately at 95 °C for 3 min. Ten microliters of solution were removed and again inspected under phase contrast microscopy for ruptured spores, which no longer appear refractive.

One to 5 μl of the TAE solution was removed and used in a standard PCR reaction (94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 45 °C for 30 sec, and 72 °C for 1 min 30 s) using primers Ambly18f and Ambly1492r (see below). The PCR product was then purified on a Qiaquick PCR purification kit (Qiagen Inc., Chatsworth, CA) and prepared for automated sequencing at the Keck Biotechnology Resource Laboratory at Yale University using all of the following microsporidia primers: Ambly18f, CACCAGGTTGATTCTGCC; SS350f, CCAAGGA(T/C)GGCAGCAGGCGCGAAA; SS350r, TTTCGCGCCTGCTGCC(G/A)TCCTTG; SS530f, GTGCCAGC-(C/A)GCCGCGG; SS530r, CCGCGG(T/G)GCTGGCAC; Ambly1047r, AACGGCCATGCACCAC; Ambly1061f, GGTGGT-GCATGGCCG; Ambly1492r, GGTTACCTTGTTACGACTT.

Sequences were aligned using the ClustalX program (Thompson, Higgins, and Gibson 1994). Aligned sequences were analyzed by maximum parsimony, neighbor joining, and maximum likelihood analyses using PAUP version 3.1b (Swofford 1993). The following species were included in the phyogenetic analysis: *Amblyospora californica* (U68473), *Amblyospora connecticus* (AF025685), *Amblyospora salinaria* (U68474), *Amblyospora stimuli* (AF027685), *Culicosporella lunata* (AF027683), *Edhazardia aedis* (AF027684), *Intrapredatorus barri* (AY013359), and *Parathelohania anophelis* (AF027682).

RESULTS

Development in *Culiseta melanura*. The first developmental stages observed in early instar larvae were small uninucleate schizonts that were mostly fusiform with the nucleus at one pole (Fig. 1, 15). These stages were observed to divide by binary fission (schizogony) (Fig. 2) and were often quite numerous in Giemsa-stained smears of newly infected larvae. In ultrastructure, schizonts were delineated by a simple plasmalemma and contained well-developed zones of Golgi apparatus within a ribosome-rich cytoplasm (Fig. 15, 16). Spindle plaques were also frequently seen in association with the nuclei of these cells (Fig. 16).

No evidence of plasmogamy was observed. However, schizonts that appeared to undergo karyokinesis to form diplokaryotic stages were regularly detected (Fig. 3, 4). These transitional stages were distinguishable in ultrastructure by the possession of two intimately associated nuclei that were separated by a weakly defined and convoluted nuclear envelope (Fig. 17). These contrasted sharply with fully formed diplokaryotic meronts, where each member of the diplokaryon was clearly defined and both were enclosed within a distinct unit membrane (Fig. 5, 18). Diplokaryotic meronts underwent synchronous binary division (merogony) to produce additional diplokarya (Fig. 6, 7, 19), which were the most commonly observed stage in second and third instar larvae. Large numbers of diplokarya were further detected within the cytoplasm of host fat body cells and the nuclei of host cells were markedly hypertrophied (Fig. 20).

Early diplokaryotic sporonts were distinguished from meronts by the possession of an amorphous outer envelope surrounding the plasmalemma, and a more vacuolated cytoplasm

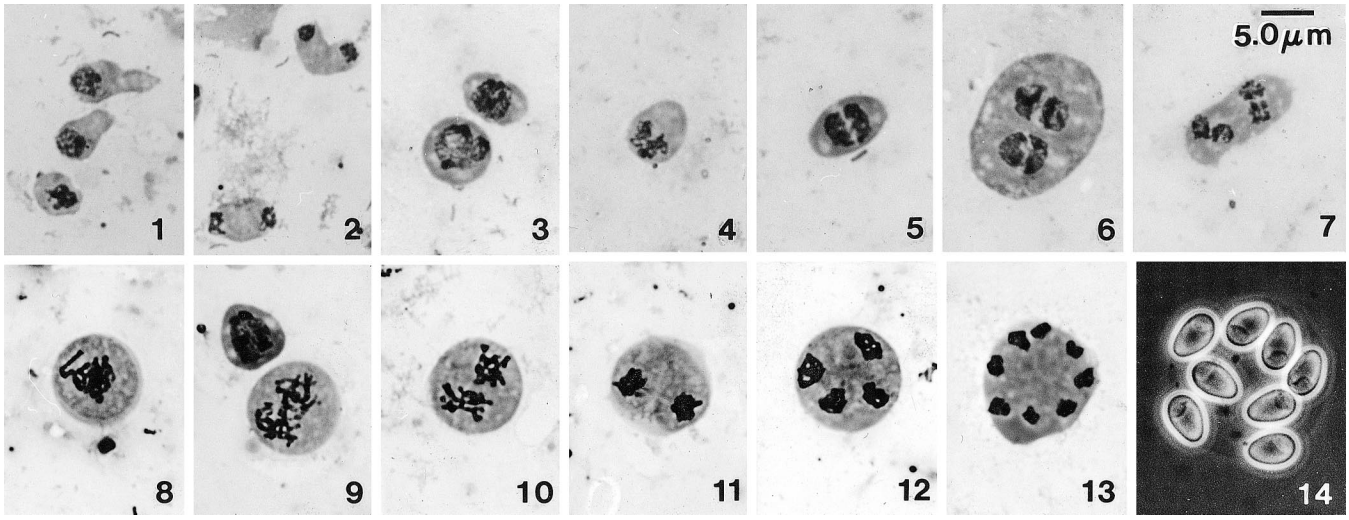

Fig. **1–14**. Developmental stages of *Hyalinocysta chapmani* from *Culiseta melanura*. Fig. **1–13,** Giemsa-stained. Fig. **14,** fresh. Bar = 5.0 μm and applies to all figures. Fig. **1.** Uninucleate schizont. Fig. **2.** Dividing schizonts. Fig. **3, 4.** Transitional schizonts undergoing karyokinesis. Fig. **5.** Diplokaryotic meront. Fig. **6, 7.** Multinucleated meronts undergoing merogony. Fig. **8.** Sporont with fused diplokarya during prophase I of meiosis. Fig. **9, 10.** Sporonts undergoing meiosis I. Fig. **11.** Binucleate sporont. Fig. **12.** Quadrinucleate sporont. Fig. **13.** Octonucleate sporont. Fig. **14.** Mature spores.

(Fig. 21). The outer envelope was of parasite origin and was interpreted as the primordium of the sporophorous vesicle. The plasmalemma was seen to pull away from the outer envelope (sporophorous vesicle) and this was accompanied by the progressive deposition of patches of dense material on the plasmalemma (Fig. 22). Granular inclusions were additionally observed within the episporontal space. These events marked the initiation of the sporogonial phase of development and were coincident with the onset of meiosis.

Meiotic division of diplokaryotic sporonts was similar to that observed in *Amblyospora*. The first evidence was the detection of synaptonemal complexes within each member of the diplokaryon (Fig. 23). A crescent-shaped organelle of unknown function, resembling the ''kinetic center'' described by Loubès (1979) and Vavra, Bai, and Panicker (1984) was additionally observed in the furrow between each member of the separating diplokaryon (Fig. 23). This was followed by dissolution of the nuclear envelopes (Fig. 24) and subsequent fusion of the two elements of the diplokaryon so as to form a single nucleus (Fig. 8). A substantial increase in the quantity and size of the granular inclusions accumulating within the episporontal space was observed at this time as well (Fig. 25). Separation of the chromosomes during meiosis I (Fig. 9, 10) resulted in the formation of a binucleated sporont with a constricted cell membrane (Fig. 11, 26). During meiosis II, quadrinuleate sporonts were formed by synchronous mitotic division of each nucleus (Fig. 12, 27, 28). This was followed by a final mitotic division producing a sporogonial plasmodium consisting of eight nuclei (Fig. 13, 29). An elaborate branched network of tubular extensions of the plasmalemma was additionally observed in octonucleate sporonts. These extensions were most prominent in the central constricted region of the plasmodium (Fig. 29, 30).

Cytokinesis of octonucleate sporonts resulted in the formation of eight uninucleate sporoblasts within the sporophorous vesicle (Fig. 31). Young sporoblasts (Fig. 32) were recognized by their thickened cell wall and highly vacuolated cytoplasm. The first organelle to be differentiated during spore morphogenesis was the polar filament, which was initially seen as an accumulation of dense vesicles within the cytoplasm and later as concentric rings with thick dense centers and borders. This was followed by the differentiation of the polaroplast, posterior vacuole, and finally the endospore and exospore walls (Fig. 33, 34). During sporogenesis, the granular and tubular inclusions within the episporontal space gradually dissipated resulting in a sporophorous vesicle void of any inclusions (Fig. 34).

Mature spores (Fig. 14) were ovoid and measured 4.49 ± 0.06 × 2.80 ± 0.05 μm (live). They were uninucleate and possessed a large posterior vacuole (Fig. 35). The polar filament was anisofilar, with 7–8 coils: 3–4 broad proximal and 4 narrow distal. The polaroplast was voluminous and occupied the anterior third of the spore. The anterior portion was lamellar with tightly compressed and regularly arranged lamellae, while the posterior portion was vesicular with wide irregularly spaced chambers (Fig. 36). The anchoring disc of the polar sac was well developed. The spore wall measured 100 nm. The exospore was laminate with two distinct layers and measured 40 nm while the endospore measured 60 nm (Fig. 37).

All stages of parasite development were found within fat body tissue as determined by histological examination of second through fourth instar larvae (n = 50). No infections of any type were detected in the gastric caeca or midgut epithelial cells. Of 20 infected fourth instar larvae examined histologically, 8 (40%) were male and 12 (60%) were female.

Development in *Orthocyclops modestus*. The earliest developmental stages observed in *O. modestus* copepods were small (~ 4 μm), oval, uninucleate schizonts (Fig. 38). Schizonts were bound by a plasmalemma that was in direct contact with the host cell cytoplasm, and contained a homogenously granular cytoplasm with numerous free ribosomes and some endoplasmic reticulum (Fig. 45). Schizogony was limited to binary division of uninucleate schizonts, giving rise to sporonts that typically possessed a nucleus at one pole (Fig. 39, 40).

The initiation of sporogony was marked by the deposition of patches of dense material on the surface of the plasmalemma and the production of a thin sporophorous vesicle (Fig. 46, 47). This was accompanied by repeated nuclear division (Fig. 41, 42) to form large (up to 20 μm), multinucleate, rosette-shaped plasmodia with up to 18 nuclei (Fig. 43). The sporophorous vesicle was seen as a thin undulating membrane that tightly enveloped the entire sporogonial plasmodium. Granular inclu-

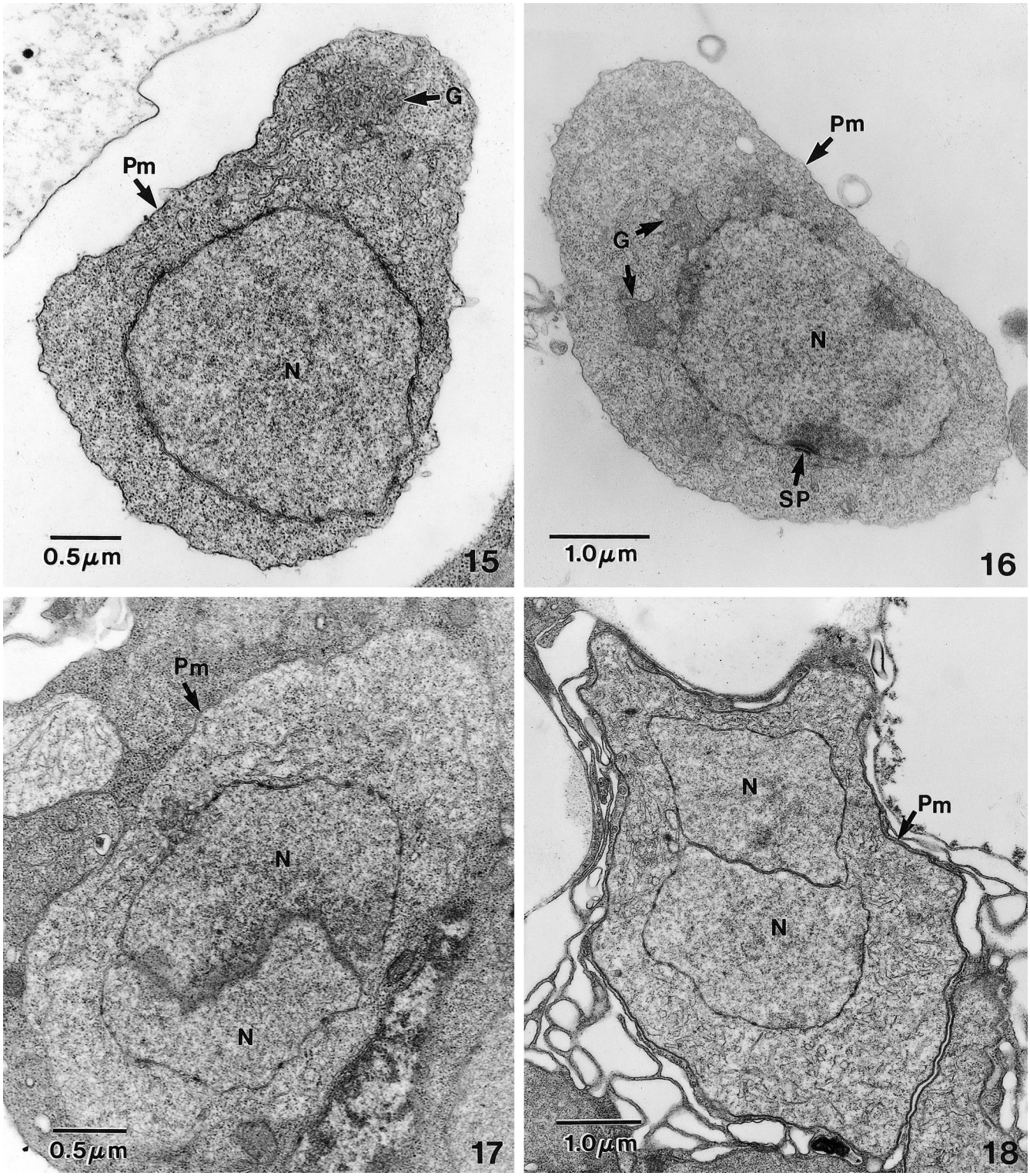

Fig. **15–18**. Electron micrographs of developmental stages of *Hyalinocysta chapmani* from *Culiseta melanura*. Fig. **15, 16.** Uninucleate schizonts. G, Golgi apparatus; N, nucleus; Pm, plasmalemma; SP, spindle plaque. Fig. **17.** Schizont undergoing karyokinesis. Fig. **18.** Diplokaryotic meront.

sions were prominent within the episporontal space and appeared as dense, homogeneous blister-like accumulations (Fig. 47–49). Short bud-like extensions of the thickened plasmalemma were occasionally noted in these sporonts (Fig. 47).

Cytoplasmic division was by budding of the sporogonial plasmodium to form unicellular sporoblasts (Fig. 43, 48). Early sporoblasts possessed a vacuolated cytoplasm and contained well-developed zones of Golgi apparatus and stacked arrays of

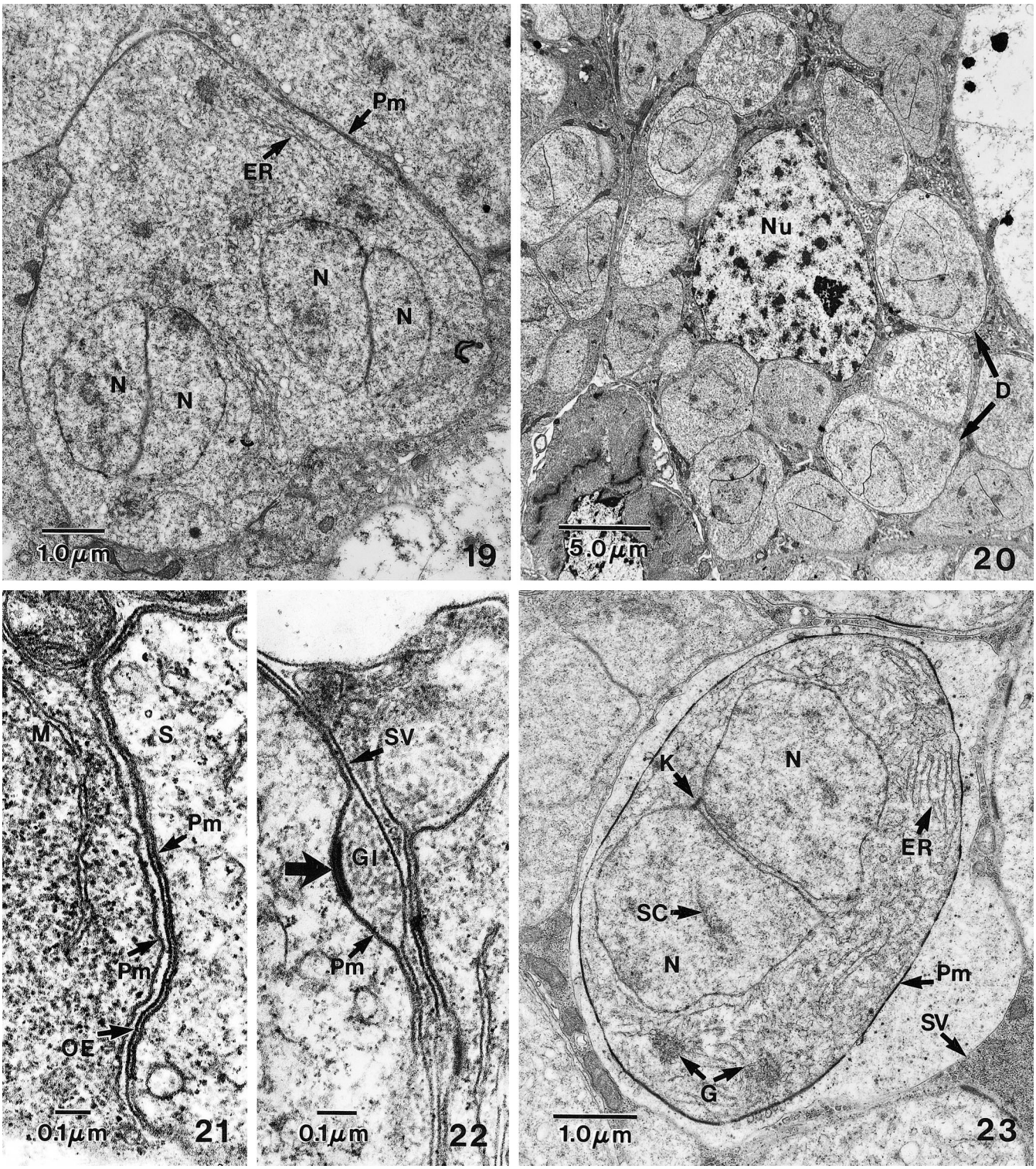

Fig. **19–23**. Electron micrographs of developmental stages of *Hyalinocysta chapmani* from *Culiseta melanura*. Fig. **19.** Dividing diplokaryotic meront. ER, endoplasmic reticulum; N, nucleus; Pm, plasmalemma. **20.** Diplokaryotic meronts (D) surrounding a hypertrophied host cell nucleus (Nu). Fig. **21.** High magnification comparison of the cytoplasm, plasmalemma (Pm), and outer envelope (OE) of a diplokaryotic meront (M) and sporont (S). Fig. **22.** High magnification of sporont showing the deposition of electron-dense material on the plasmalemma (arrow) and separation of the sporophorous vesicle (SV). GI, granular inclusions. Fig. **23.** Diplokaryotic sporont during initial stage of meiosis showing synaptonemal complexes (SC) and a crescent shaped kinetic center (K) connecting each member of the diplokaryon (N). G, Golgi apparatus.

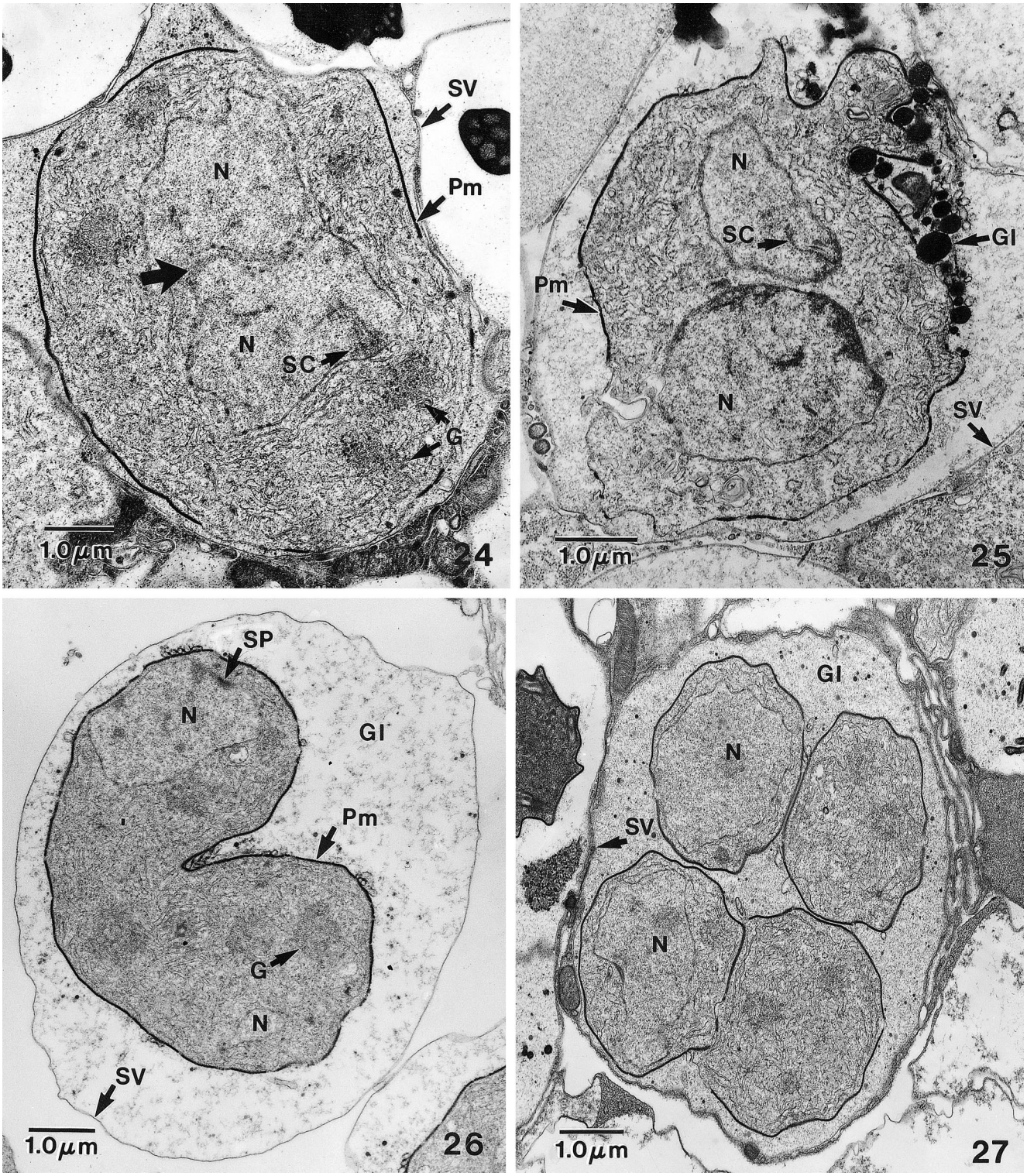

Fig. **24–27**. Electron micrographs of developmental stages of *Hyalinocysta chapmani* from *Culiseta melanura*. Fig. **24, 25.** Diplokaryotic sporonts during the early phase of meiosis. Note dissolution of the nuclear envelopes in Fig. 24 (large arrow). G, Golgi apparatus; GI, Granular inclusions; N, nucleus; Pm, plasmalemma, SC, synaptonemal complex; SV, sporophorous vesicle. Fig. **26.** Binucleate sporont. SP, spindle plaque. Fig. **27.** Quadrinucleate sporont.

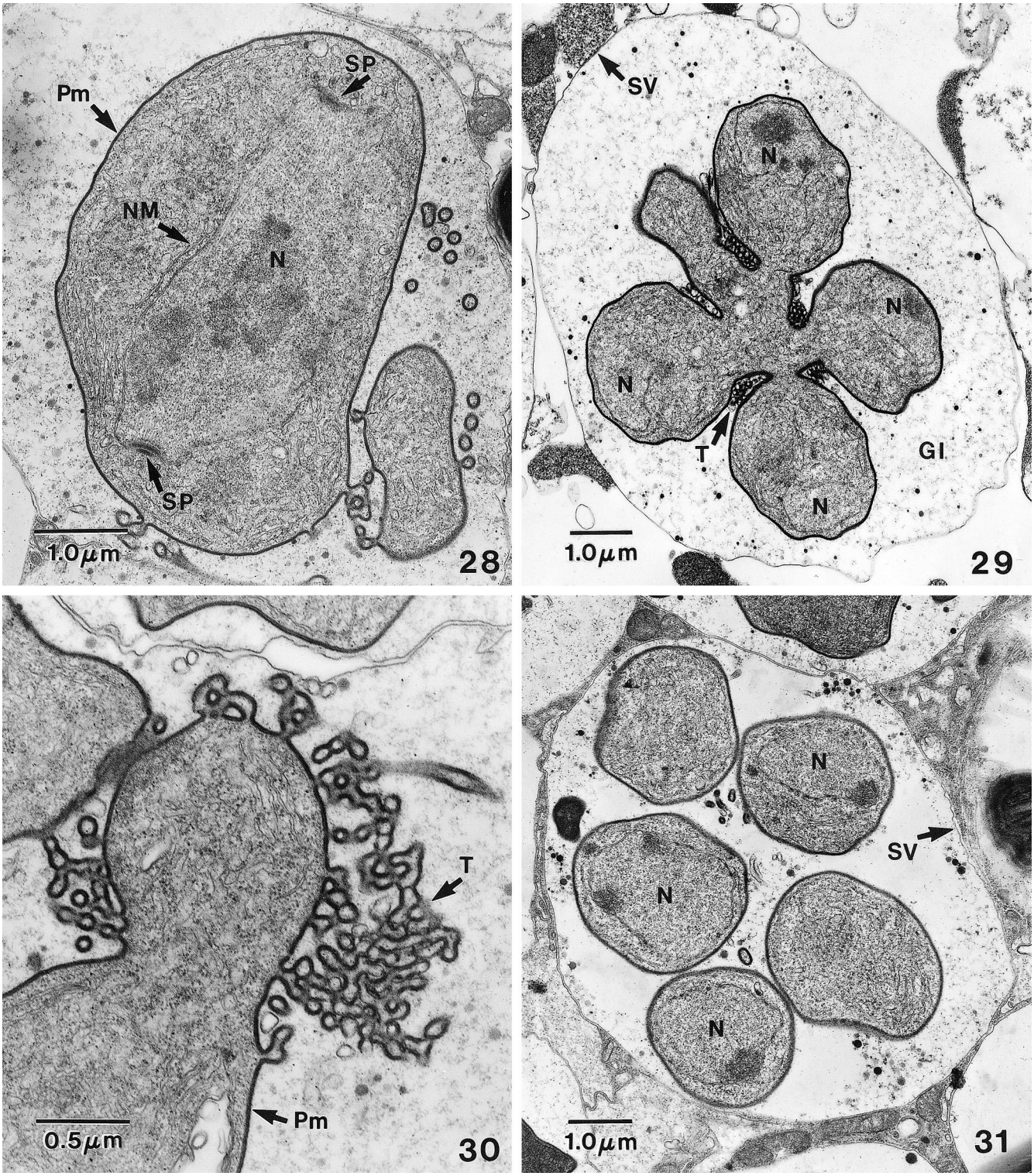

Fig. **28–31**. Electron micrographs of developmental stages of *Hyalinocysta chapmani* from *Culiseta melanura*. Fig. **28.** Dividing sporont. N, nucleus; NM, nuclear membrane. Pm, plasmalemma, SP, spindle plaque. Fig. **29.** Section through an octonucleate sporont plasmodium. GI, Granular inclusions; SV, sporophorous vesicle; T, tubules. Fig. **30.** Tubular extensions (T) of an octonucleate sporont. Fig. **31.** Early sporoblasts.

endoplasmic reticulum (Fig. 48, 49). The sequence of organelle formation during sporogenesis was similar to that observed in meiospores, with initial differentiation of the polar filament (Fig. 50), followed by the polaroplast, posterior vacuole, and extrusion apparatus, and lastly the endospore and exospore wall (Fig. 51). During sporogenesis, the granular inclusions within the episporontal space and the sporophorous vesicle gradually degenerated.

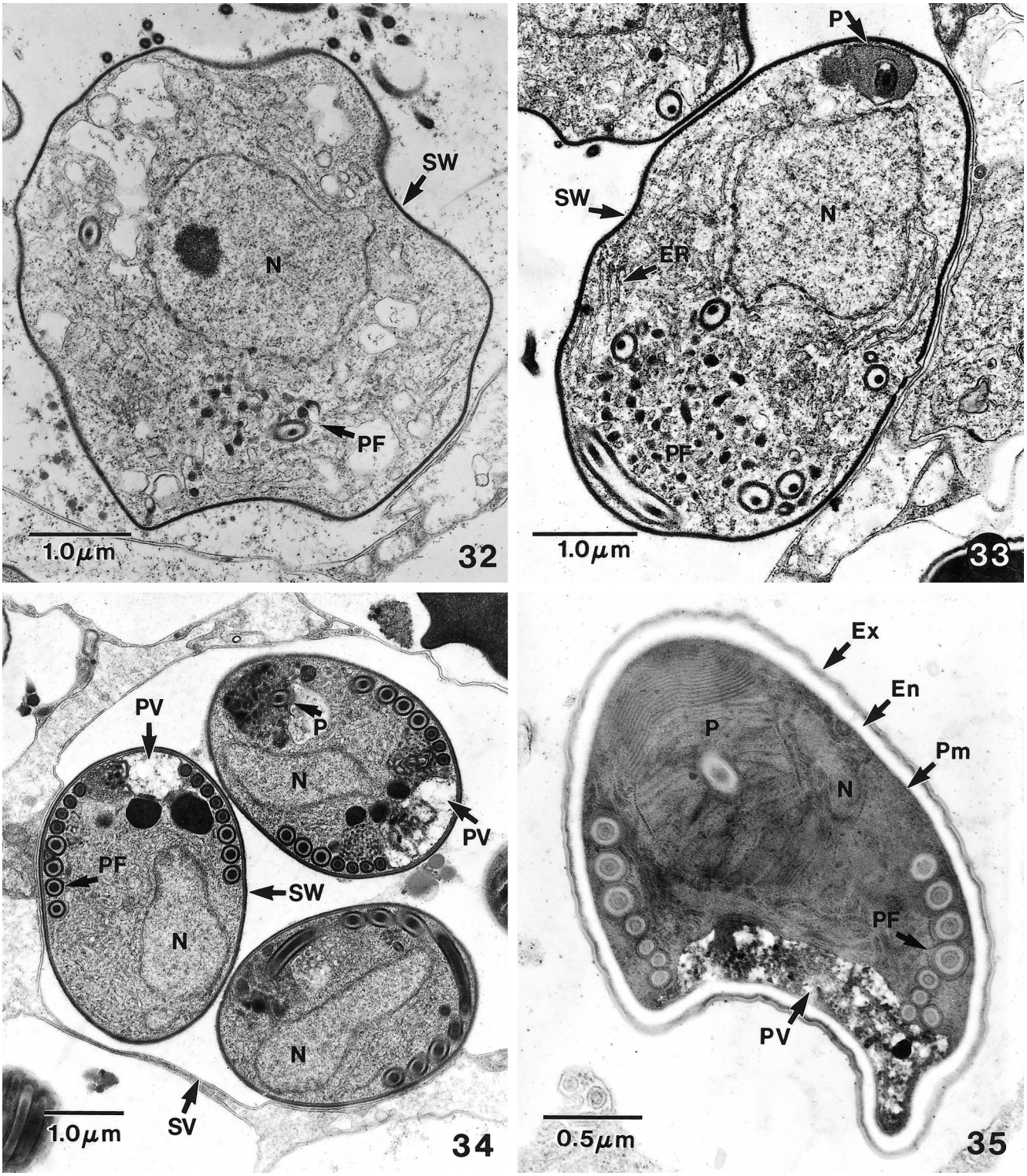

Fig. **32–35**. Electron micrographs of developmental stages of *Hyalinocysta chapmani* from *Culiseta melanura*. Fig. **32–34.** Developing sporoblasts. ER, endoplasmic reticulum; N, nucleus; P, polaroplast; PF, polar filament; Pm, plasmalemma; PV, posterior vacuole; SV, sporophorous vesicle; SW, spore wall.Fig. **35.** Mature meiospore. En, endospore; Ex, exospore.

Mature spores (Fig. 44) were ovoid and averaged 5.29 ± 0.04 × 3.54 ± 0.14 μm (live). They were uninucleate and possessed a large posterior vacuole (Fig. 52). The polaroplast occupied the interior two-thirds of the spore and was vesicular with large irregularly spaced chambers on the anterior end and small tightly compressed honeycomb chambers on the posterior end (Fig. 52). The polar filament was isofilar with 6–7 coils, and the anchoring disc of the polar sac was well developed (Fig. 51).

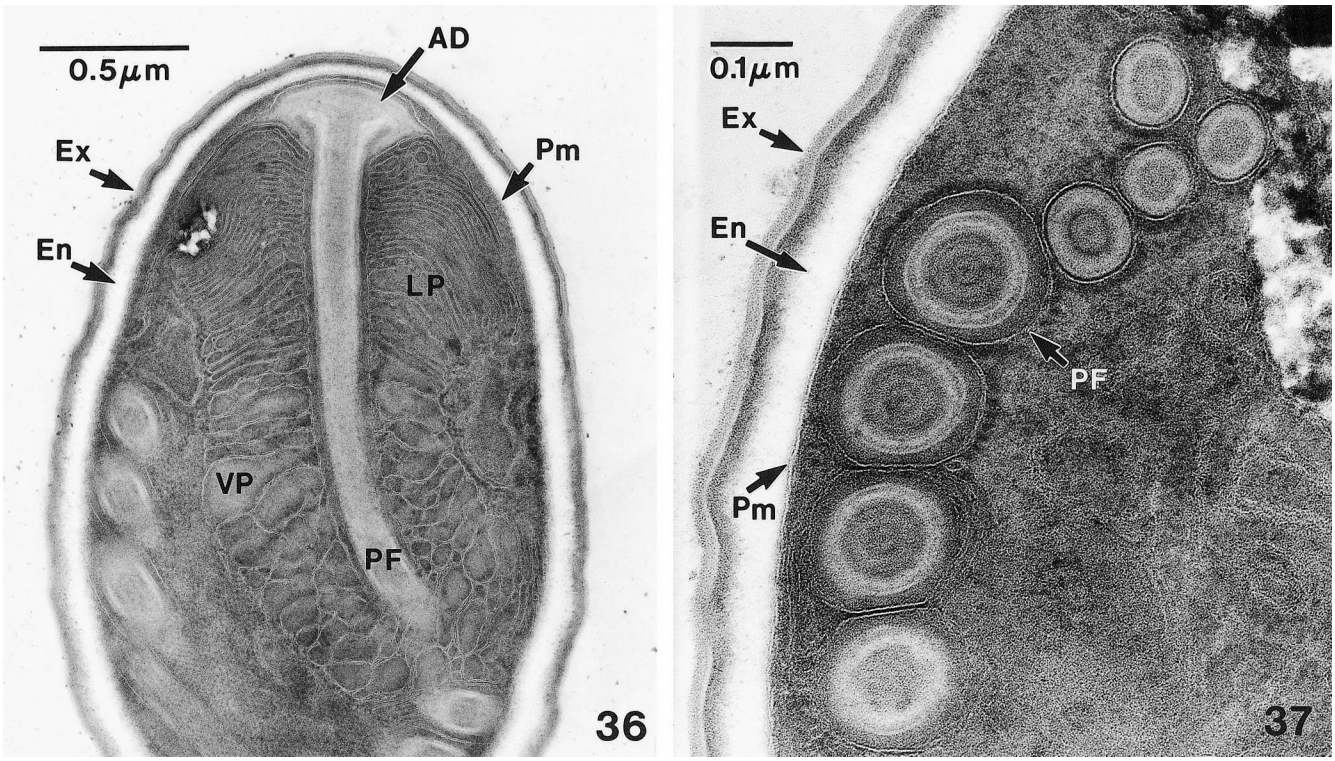

Fig. **36–37**. Electron micrographs of mature spores of *Hyalinocysta chapmani* from *Culiseta melanura*. AD, anchoring disc; En, endospore; Ex, exospore; LP, lamellar polaroplast; PF, polar filament; Pm, plasmalemma; VP, vesicular polaroplast.

The spore wall measured 70 nm; the exospore was approximately 15 nm while the endospore measured 55 nm.

Natural infections were most commonly found in adult female stages of *O. modestus* in nature. However, 3 adult males were additionally found to be infected in Giemsa-stained smears. All stages of parasite development in the female host were confined to the median ovary and paired lateral oviducts. These tissues became grossly distended with mature spores and this eventually resulted in death of the copepod. Infections appeared to inhibit egg development as no infected egg sac-bearing females were ever detected in the field. The specific tissues infected in males could not be determined from the Giemsa-stained smears and naupli and copepodid stages were not examined.

Transmission studies. Results of the laboratory transmission studies are summarized in Table 1. *Hyalinocysta chapmani* was orally transmitted to *C. melanura* larvae in 6 of 7 separate bioassays conducted during the year where larvae were exposed to fresh spores obtained from field-collected *O. modestus* copepods. Infection rates ranged from 10.7–26.0% and averaged 14.1%. Developmental stages associated with these lab-induced infections were identical to those observed in field-collected specimens: all infected individuals developed typical fat body infections that culminated in the formation of meiospores. Meiospore infections were also detected in 3.9% (14/356) of adult males, and 1.8% (6/332) of adult females that successfully emerged. These prevalences were not significantly different ($P = 0.15$, chi-square analysis) between the two sexes. No ''early'' or ''binucleate'' spores, as reported in *Amblyospora* or *Edhazardia*, were detected in any host stage nor were infections found in any tissue other than fat body. Egg rafts were obtained from 135 female *C. melanura* that were exposed as larvae to spores from *O. modestus*. No infections were found in any of these females. Hatching progeny were obtained from 35 of these egg rafts and all were uninfected upon examination.

Due to the limited availability of field-collected copepods, only 2 feeding experiments were conducted with *O. modestus* and meiospores from *C. melanura*. Infection rates averaged

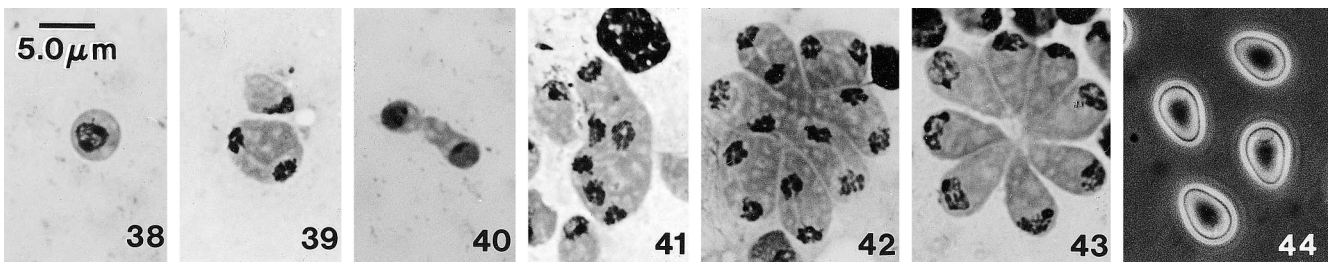

Fig. **38–44**. Developmental stages of *Hyalinocysta chapmani* from *Orthocylops modestus*. Fig. **38–43,** Giemsa-stained. Fig. **43,** live. Bar = 5.0 μm and applies to all figures. Fig. **38.** Uninucleate schizont. Fig. **39, 40.** Schizonts undergoing binary division (schizogony). Fig. **41, 42.** Sporonts undergoing karyokinesis (sporogony). Fig. **43.** Sporogonial plasmodium. Fig. **44.** Mature spores.

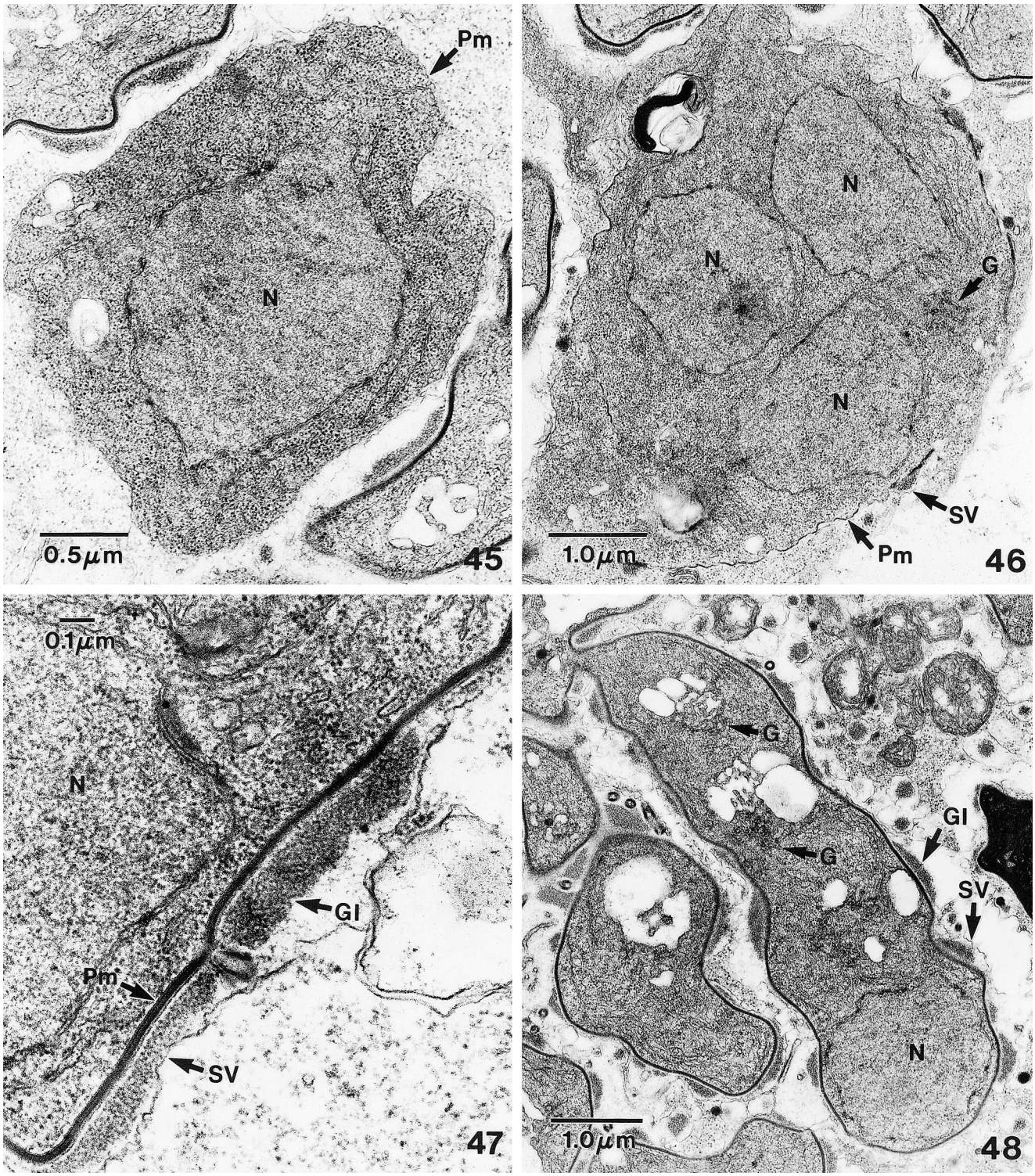

Fig. **45–48**. Electron micrographs of developmental stages of *Hyalinocysta chapmani* from *Orthocyclops modestus*. Fig. **45.** Uninucleate schizont. N, nucleus; Pm, plasmalemma. Fig. **46.** Early multinucleated sporont. G, Golgi apparatus; SV, sporophorous vesicle. Fig. **47.** High magnification view of sporont plasmalemma (Pm) and sporophorous vesicle (SV). GI, granular inclusions. Fig. **48.** Finger-like projection of a sporogonial plasmodium just prior to cytoplasmic cleavage.

48% and were much higher than those achieved in the mosquito feeding trials. The infections and developmental sequences were identical to those observed in field-collected specimens.

No infections of any type were obtained in feeding trials where *C. melanura* larvae (n = 150) were exposed to meiospores, and female *O. modestus* (n = 50) were exposed to uninucleate spores harvested from other copepods. We did not conduct transmission tests with other copepod or mosquito species.

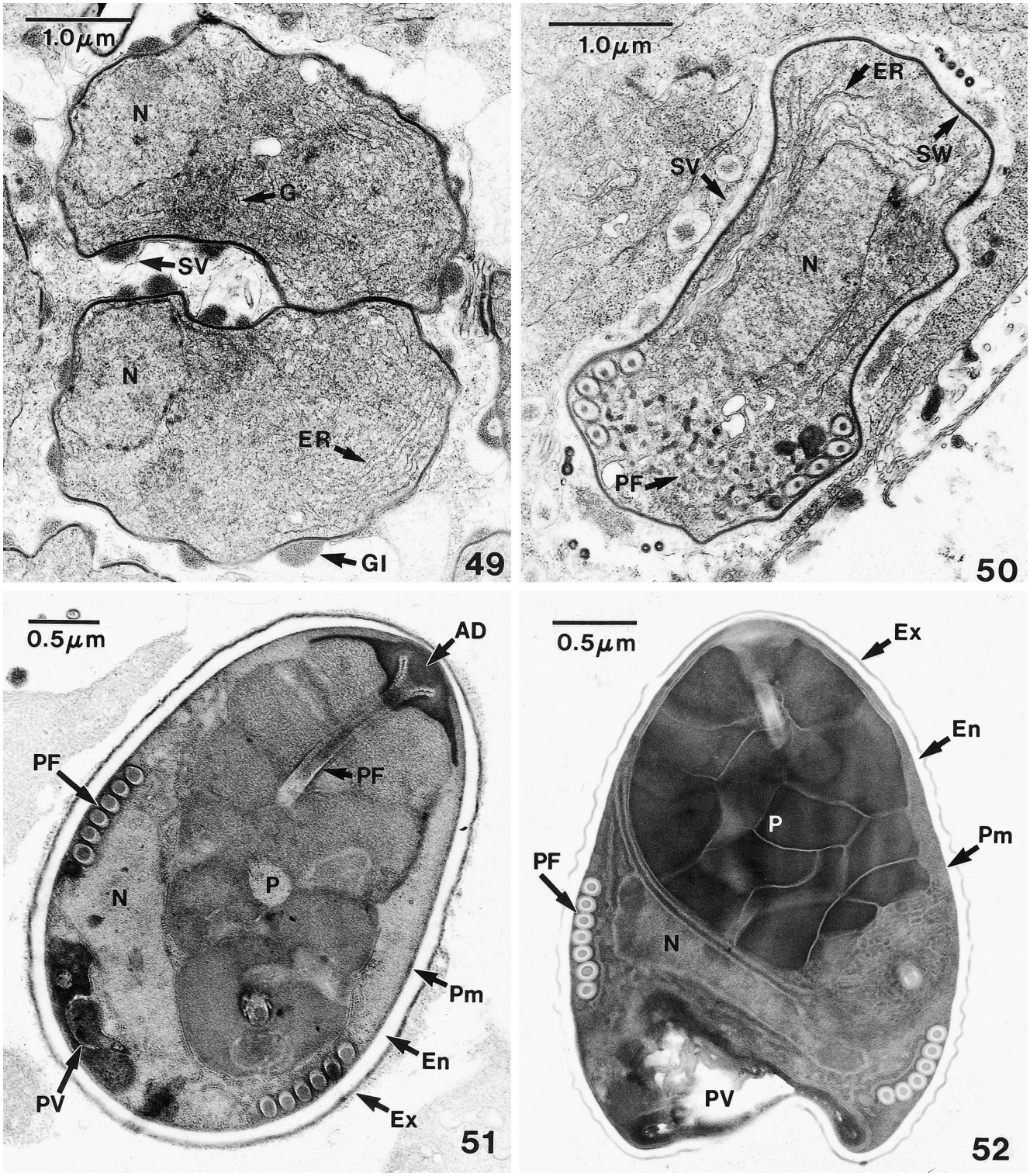

Fig. **49–52**. Electron micrographs of developmental stages of *Hyalinocysta chapmani* from *Orthocyclops modestus*. Fig. **49.** Early sporoblasts. ER, endoplasmic reticulum; G, Golgi apparatus; GI, granular inclusions; N, nucleus; SV, sporophorous vesicle. Fig. **50.** Later stage sporoblast. PF, polar filament; SW, spore wall. Fig. **51.** Mature sporoblast. En, endospore; Ex, exospore; P, polaroplast; Pm, plasmalemma, PV, posterior vacuole. Fig. **52.** Mature spore.

Table 1. Results of bioassay experiments showing the infectivity of spores of *Hyalinocysta chapmani* harvested from naturally infected *Orthocyclops modestus* copepods and *Culiseta melanura* larvae to laboratory-reared second instar *C. melanura* and field-collected adult female *O. modestus*, respectively.

Target host	Spore source	Date of collection	Number of target hosts exposed	% infection
C. melanura	*O. modestus*	May	100	12.0
		June	150	10.7
		July	200	18.5
		August	50	26.0
		September	100	11.0
		October	150	16.0
		November	50	0
Total and average			800	14.1
O. modestus	*C. melanura*	May	50	30.0
		June	50	66.0
Total and average			100	48.0

Molecular phylogeny. The small subunit rDNA sequence of *H. chapmani* isolated from meiospores from the mosquito host, *C. melanura*, was identical to the small subunit rDNA sequence obtained from spores from the copepod host, *O. modestus*. This finding corroborated the laboratory transmission studies and confirmed the intermediary role of *O modestus* in the life cycle of this microsporidium. Phylogenetic analyses with eight related microsporidia from mosquitoes (Fig. 53) showed *H. chapmani* to be significantly different from the *Amblyospora* species and the other related taxa. The *Amblyospora* and *Edhazardia* species presented here are representatives from the *Culex*, *Ochlerotatus*, and *Aedes* groups and represent the diversity of the true *Amblyospora* species. Neighbor joining and maximum likelihood analysis gave identical tree topologies and placed *H. chapmani* as a sister group to *Culicosporella lunata* (Hazard & Savage, 1970) Weiser, 1977 (host = *Culex pilosis*).

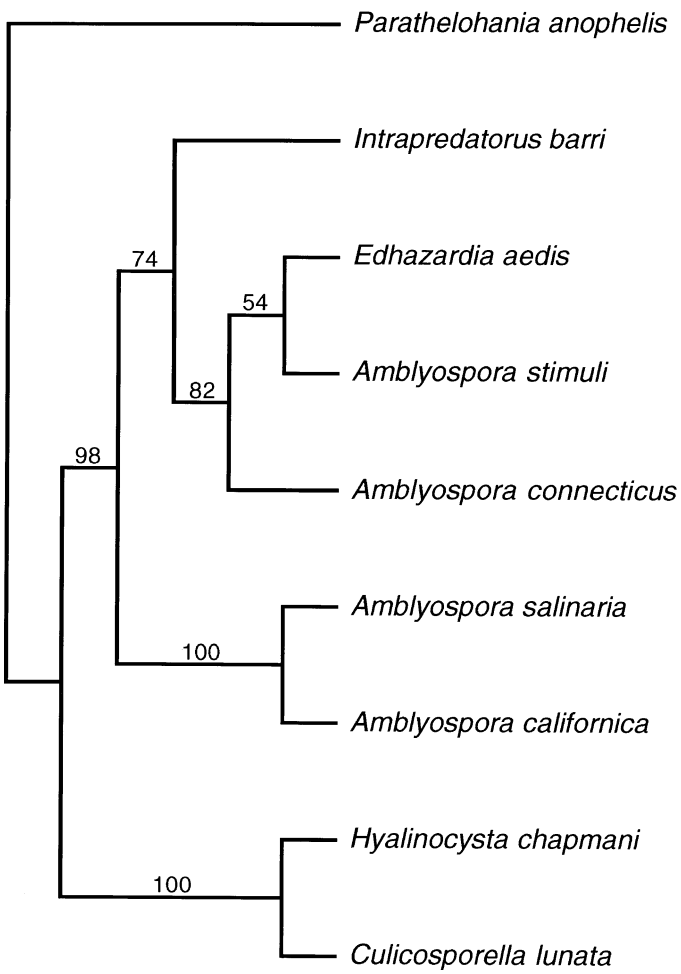

Fig. **53**. Bootstrap analysis using 100 replicates of the branch and bound search method, optimality criterion = maximum parsimony. Neighbor joining and maximum likelihood analysis gave similar tree topologies.

DISCUSSION

Results reported herein conclusively document the intermediary role of the copepod, *O. modestus* in the life history of *H. chapmani*. The identical rDNA sequences of the spores isolated from each host provide further confirmation and reaffirm the effectiveness of using rDNA sequence data for identifying potential intermediate hosts (Vossbrinck, Andreadis, and Debrunner-Vossbrinck 1998). This now brings to four the number of microsporidian genera from which a copepod intermediate host has been described. The others, also from mosquitoes, include: *Amblyospora* (10 species) (Andreadis 1985a, 1999; Becnel 1992; Becnel and Andreadis 1998; Micieli, Garcia, and Becnel 1998, 2000a, 2000b; Sweeney, Hazard, and Graham 1985; Sweeney, Doggett, and Piper 1990; White, Fukuda, and Undeen 1994); *Duboscqia* (1 species) (Sweeney, Doggett, and Piper 1993); and *Parathelohania* (1 species) (Avery and Undeen 1990). The phylogenetic analysis additionally supports the preservation of *Hyalinocysta* and *Culicosporella* as separate and distinct genera.

Our interpretation of the complete life cycle of *H. chapmani* in *C. melanura* and *O. modestus* is summarized in Fig. 54 and contrasted with the life cycles of other closely related mosquito-parasitic microsporidia below. Infections are initiated in larval *C. melanura* following the oral ingestion of uninucleate spores that are released into the aquatic environment with the death of infected copepods. Spores germinate within the lumen of the midgut but unlike *Amblyospora* spp. (Andreadis 1985b, 1988b) and *Edhazardia aedis* (Becnel et al. 1989; Johnson, Becnel, and Undeen 1997), *H. chapmani* does not initially become established within the epithelial cells of the midgut or gastric caeca, but rather directly invades fat body tissue where all subsequent development occurs. These observations lead us to infer that the everted polar filament of the ingested spore must pass through the epithelial cells of the gut and inoculate the sporoplasm directly into fat body cells to initiate infection. The absence of early autoinfective spores that function in dispersal of infection from the gastric caeca to other tissues as noted in *E. aedis* (Johnson, Becnel, and Undeen 1997), further supports this conclusion.

Uninucleated schizonts undergo binary division during an initial schizogonic cycle. This results in increased numbers of uninucleated stages that then undergo karyokinesis to form diplokaryotic meronts. The process by which the diplokaryotic condition is achieved in *H. chapmani* appears to be unique and is markedly different from that reported in *Culicospora magna* (Kudo, 1920) Weiser, 1977 (Becnel et al. 1987), *E. aedis* (Becnel et al. 1989), *Hazardia milleri* (Hazard & Fukuda, 1974) Weiser 1977 (Hazard, Fukuda, and Becnel 1985), and all spe-

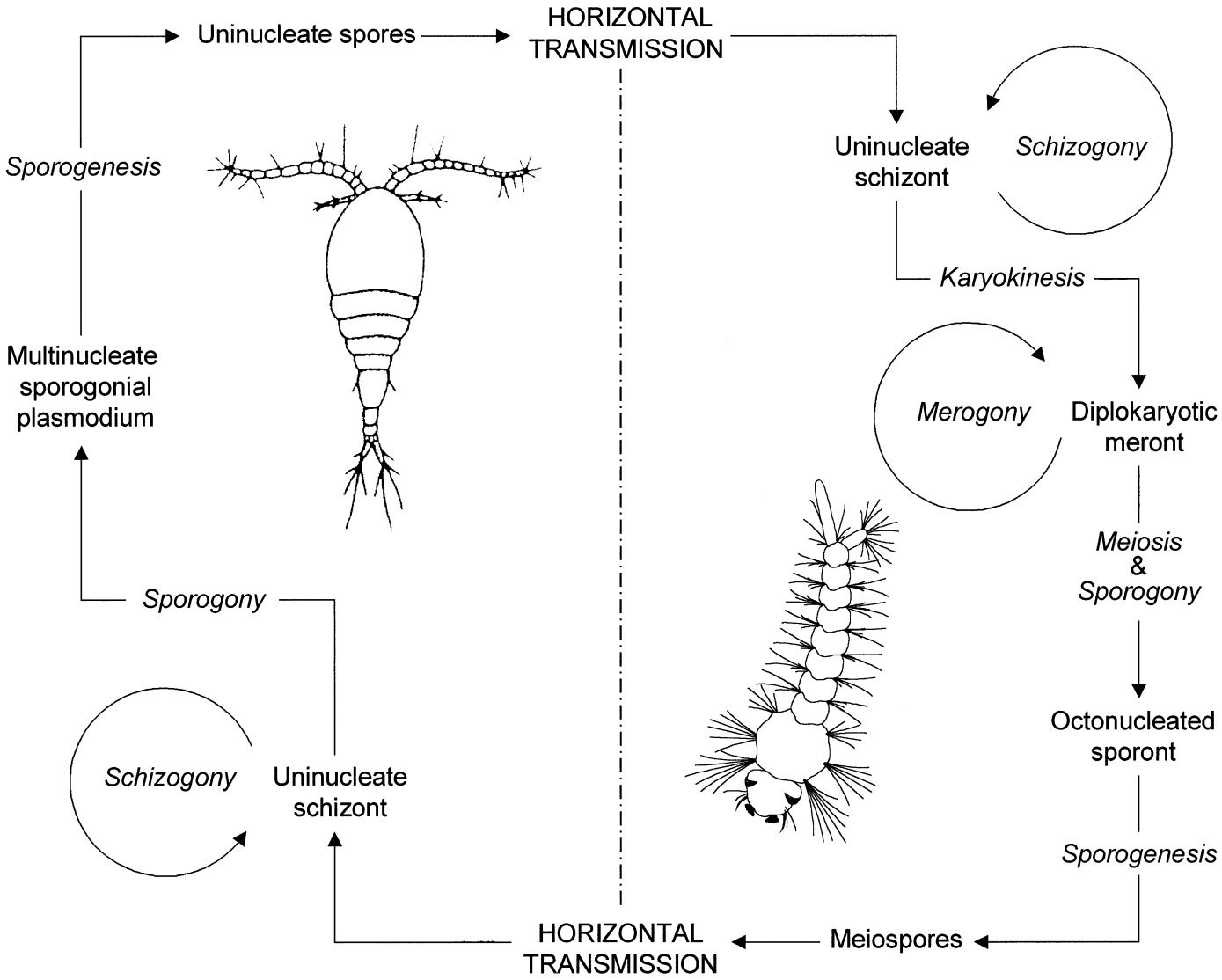

Fig. **54**. Life cycle of the microsporidium *Hyalinocysta chapmani* in the mosquito *Culiseta melanura* and the copepod *Orthocyclops modestus*.

cies of *Amblyospora* that have been studied (*A. californica, A. connecticus, A. salinaria, A. stimuli*) (Andreadis 1985b, 1988b; Becnel 1992; Becnel and Andreadis 1998). In these microsporidia, diplokaryotic meronts are formed by cytoplasmic pairing (plasmogamy) and nuclear association of uninucleated gametes. In *C. magna* (Becnel et al. 1987), *E. aedis* (Becnel et al. 1989; Johnson, Becnel, and Undeen 1997), and *Amblyospora californica* (Kellen & Lipa, 1960) Hazard & Oldacre, 1975 (Becnel 1992), uninucleated gametes, which undergo plasmogamy are further distinguished ultrastructurally by the possession of a nipple-like structure on the anterior end of the plasmalemma. No such structures or stages were observed in *H. chapmani*.

Diplokaryotic meronts enter a second proliferative phase of merogony during which large numbers are produced by synchronous binary division. 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 octonucleated 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 *H. chapmani* are virtually identical to those reported in *Amblyospora* spp. (Andreadis 1983; Andreadis and Hall 1979; Becnel and Sweeney 1990; Micieli, Garcia, and Becnel 2000a; Sweeney, Graham, and Hazard 1988; Vavra, Bai, and Panicker 1984) and *C. lunata* (Becnel and Fukuda 1991). The repeated detection of synaptonemal complexes in each member of the diplokaryon prior to nuclear fusion continues to be problematic, as it is unclear how the diploid state, evidenced by the occurrence of paired homologous chromosomes, is achieved in each nucleus. This aspect of the life cycle has been reviewed by Flegel and Pasharawipes (1995) who argue that the occurrence of synaptonemal complexes in unfused diplokaryotic nuclei in *Amblyospora* represents a tandem ‘‘pseudomeiosis’’ that stalls at diakinesis. They hypothesize that stalled development occurs when conflicts arise between meront intracellular factors (e.g. ploidy, position with respect to start) and extracellular factors (e.g. host sex, host cell type, host hormones) that directly influence which of four developmental pathways diplokaryotic meronts may take in the larval mosquito host. This hypothesis is based on the assumption that diplokaryotic meronts harbor all the genetic

information necessary for each developmental sequence, and is supported in part by the polymorphic nature of *Amblyospora* in the mosquito host. A similar effect could occur with diplokaryotic meronts of *H. chapmani*, which are capable of undergoing merogony (mitotic division) and/or meiosis and sporogony. It is unclear however, what triggers these events in the life cycle and whether daughter cells produced during the merogonial cycle are the only stages capable of initiating the meiotic sequence.

Most infected larvae die during the fourth stadium. Some infected individuals survive to adulthood, apparently due to a less intense infection. However there is no evidence of further parasite development that might lead to either maternal- or paternal-mediated vertical transmission.

With the exception of the size and shape of the spores and number of nuclei in the rosette-shaped sporogonial plasmodia, development of *H. chapmani* in *O. modestus* was very similar to that described in each of the other three genera (*Amblyospora*, *Duboscqia*, and *Parathelohania*) that utilize intermediate copepod hosts. *Hyalinocysta chapmani* is horizontally transmitted to *O. modestus* copepods via oral ingestion of extracellular meiospores that are released into the water with the death of infected mosquito larvae. Infections become established within ovarian tissue of female copepods and all parasite development is haplophasic. Uninucleate schizonts derived from germinated spores divide by binary division during an initial schizogonic cycle. Newly formed uninucleate cells produce a thin sporophorous vesicle and undergo repeated nuclear division during sporogony to produce a rosette-shaped, multinucleated sporogonial plasmodium with up to 18 nuclei. This is followed by cytoplasmic cleavage, sporogenesis, and disintegration of the sporophorous vesicle to form membrane-free uninucleate spores. Infected females eventually die without producing eggs.

The most significant variation in the life cycle of *H. chapmani*, when compared to other mosquito-parasitic microsporidia, is its sole reliance on horizontal transmission and absence of a developmental sequence leading to transovarial transmission in the mosquito host. The latter is a universal theme observed with all other polymorphic groups although the transmission routes are unknown for *Intrapredatorus barri* (Chen, Kuo, and Wu 1998). Transovarial transmission is generally thought to be the single most important adaptation for survival that has evolved within the mosquito-parasitic species (Lucarotti and Andreadis 1995). The evolutionary significance of relying on horizontal transmission via an intermediate host in *H. chapmani* is most probably due to the natural ecology of each host. Both hosts develop in close association with one another in subterranean crypts in permanent fresh water swamps. These sites generally contain water throughout the year and copepods are omnipresent. *Culiseta melanura* overwinters as a larva, is multivoltine, and has overlapping generations. Larvae in all stages of development can be found from March through November (TGA, pers. observ.). The omnipresence of both hosts therefore affords abundant opportunity for continuous horizontal transmission throughout much of the year.

Analysis of the molecular phylogeny data (Fig. 53) further suggests that transovarial transmission and the developmental sequence leading to ovarian infection may have been secondarily lost in *H. chapmani*, as they occur in all other closely related genera (*Amblyospora*, *Edhazardia*, *Culicosporella* and *Culicospora*) including our outgroup *Parathelohania*. It has become increasingly evident that characters representing ecological states (e.g. intermediate host) can be gained and lost over relatively short evolutionary time (Baker et al. 1997, 1998; Weiss and Vossbrinck 1999). It is therefore our view that the ancestral state for these mosquito-parasitic microsporidia included transovarial transmission as well as an intermediate host.

TAXONOMIC SUMMARY

Hyalinocysta chapmani Hazard and Oldacre, 1975
Amended Diagnosis

Type definitive host. *Culiseta melanura* Coquillett (Diptera: Culicidae)

Type intermediate host. *Orthocyclops modestus* (Herrick) (Copepoda: Cyclopidae)

Transmission. Per os to *C. melanura* larvae via uninucleate spores from *O. modestus*. Per os to *O. modestus* females via meiospores from *C. melanura* larvae.

Site of infection. Adipose tissue of *C. melanura*. Ovarian tissue of female *O. modestus*.

Interface. In *C. melanura*, schizonts and meronts contiguous with host cell cytoplasm. Sporophorous vesicle formed by diplokaryotic sporont during sporogony; persistent through sporogenesis. In *O. modestus*, schizonts contiguous with host cell cytoplasm. Sporophorous vesicle formed by multinucleated sporont during sporogony; non-persistent through sporogenesis.

Development. Uninucleate spores from *O. modestus* ingested by *C. melanura* larvae to initiate infection. Schizogony by binary division of uninucleate schizonts followed by karyokinesis to form diplokaryotic meronts. Merogony by multiple division of diplokarya. Sporogony involves meiosis and sporophorous vesicle formation leading to the production of eight uninucleate meiospores. Meiospores ingested by *O. modestus* to initiate infection. Schizogony by binary division of uninucleate schizonts followed by sporogony and sporophorous vesicle formation to produce rosette-shaped multinucleate plasmodia and free uninucleate spores.

Spores. Meiospores from *C. melanura* uninucleate; ovoid; $4.49 \pm 0.06 \times 2.80 \pm 0.05$ μm (live); polar filament anisofilar with 7–8 coils (3–4 broad proximal and 4 narrow distal); polaroplast bipartite with proximal part lamellar and distal part vesicular; posterior vacuole prominent; exospore laminate with two layers, 40 nm; endospore 60 nm. Spores from *O. modestus* uninucleate; ovoid; $5.29 \pm 0.04 \times 3.54 \pm 0.14$ μm (live); polar filament isofilar with 6–7 coils; polaroplast bipartite with proximal part vesicular and distal part honeycomb-like; posterior vacuole prominent; exospore 15 nm; endospore 55 nm.

Type locality. *C. melanura*, near Kinder, Louisiana, USA. *O. modestus*, Cornwall, Litchfield County, Connecticut, USA. Additional locality, Chester, Middlesex County, Connecticut, USA.

Deposition of type specimens. Type slides have been deposited in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington DC, USA. *Culiseta melanura*: holotype (USNM 24391), paratype (USNM 24392), voucher specimen (USNM 1003477). *Orthocyclops modestus*: hapantotype (USNM 1003480). Additional slides and type specimens embedded in plastic resin are also in the collection of the senior author.

ACKNOWLEDGMENTS

We thank Collen Scott and John Shepard for technical assistance in the laboratory and field and Michael Thomas for preparation of figures. This research was supported in part by USDA/CSREES NRI Grant 95–37312–1687.

LITERATURE CITED

Andreadis, T. G. 1983. Life cycle and epizootiology of *Amblyospora* sp. (Microspora: Amblyosporidae) in the mosquito, *Aedes cantator*. *J. Protozool.*, **30**:509–518.

Andreadis, T. G. 1985a. Experimental transmission of a microsporidian pathogen from mosquitoes to an alternate copepod host. *Proc. Nat. Acad. Sci. USA,* **82**:5574–5577.

Andreadis, T. G. 1985b. Life cycle, epizootiology and horizontal transmission of *Amblyospora* (Microspora: Amblyosporidae) in a univoltine mosquito, *Aedes stimulans*. *J. Invertebr. Pathol.,* **46**:31–46.

Andreadis, T. G. 1988a. Comparative susceptibility of the copepod, *Acanthocyclops vernalis* to a microsporidian parasite, *Amblyospora connecticus* from the mosquito, *Aedes cantator*. *J. Invertebr. Pathol.,* **52**:73–77.

Andreadis, T. G. 1988b. *Amblyospora connecticus* sp. nov. (Microsporida: Amblyosporidae): horizontal transmission studies in the mosquito, *Aedes cantator* and formal description. *J. Invertebr. Pathol.,* **52**:90–101.

Andreadis, T. G. 1999. Epizootiology of *Amblyospora stimuli* (Microsporidiida: Amblyosporidae) infections in field populations of a univoltine mosquito, *Aedes stimulans* (Diptera: Culicidae), inhabiting a temporary vernal pool. *J. Invertebr. Pathol.,* **74**:198–205.

Andreadis, T. G. & Hall, D. W. 1979. Development, ultrastructure, and mode of transmission of *Amblyospora* sp. (Microspora: Thelohaniidae) in the mosquito. *J. Protozool.,* **26**:444–452.

Avery, S. W. & Undeen, A. H. 1990. Horizontal transmission of *Parathelohania anophelis* to the copepod, *Microcyclops varicans*, and the mosquito, *Anopheles quadrimaculatus*. *J. Invertebr. Pathol.,* **56**: 98–105.

Baker, M. D., Vossbrinck, C. R., Becnel, J. J. & Andreadis, T. G. 1998. Phylogeny of *Amblyospora* (Microsporidia: Amblyosporidae) and related genera based on small subunit ribosomal DNA data: a possible example of host parasite speciation. *J. Invertebr. Pathol.,* **71**:199–206.

Baker, M. D., Vossbrinck, C. R., Becnel, J. J. & Maddox, J. V. 1997. Phylogenetic position of *Amblyospora* Hazard & Oldacre (Microspora: Amblyosporidae) based on small subunit rRNA data and its implication for the evolution of the microsporidia. *J. Eukaryot. Microbiol.,* **44**:220–225.

Becnel, J. J. 1992. Horizontal transmission and subsequent development of *Amblyospora californica* (Microsporida: Amblyosporidae) in the intermediate and definitive hosts. *Dis. Aquat. Org.,* **13**:17–28.

Becnel, J. J. & Andreadis, T. G. 1998. *Amblyospora salinaria* n. sp. (Microsporidia: Amblyosporidae): parasite of *Culex salinarius* (Diptera: Culicidae), its life stages in an intermediate host and establishment as a new species. *J. Invertebr. Pathol.,* **71**:258–262.

Becnel, J. J. & Fukuda, T. 1991. Ultrastructure of *Culicosporella lunata* (Microsporida: Culicosporellidae fam. n.) in the mosquito *Culex pilosis* (Diptera: Culicidae) with new information on the developmental cycle. *Europ. J Protistol.,* **34**:313–322.

Becnel, J. J. & Sweeney, A. W. 1990. *Amblyospora trinus* n. sp. (Microsporida: Amblyosporidae) in the Australian mosquito *Culex halifaxi* (Diptera: Culicidae). *J. Protozool.,* **37**:584–592.

Becnel, J. J., Hazard, E. I., Fukuda, T. & Sprague, V. 1987. Life cycle of *Culicospora magna* (Kudo, 1920) (Microsporida: Culicosporidae) in *Culex restuans* Theobald with special reference to sexuality. *J. Protozool.,* **34**:313–322.

Becnel, J. J., Sprague, V., Fukuda, T. & Hazard, E. I. 1989. Development of *Edhazardia aedis* (Kudo, 1930) n. g., n. comb. (Microsporida: Amblyosporidae) in the mosquito *Aedes aegypti* (L.) (Diptera: Culicidae). *J. Protozool.,* **36**:119–130.

Chen, W. J., Kuo, T. L. & Wu, S. T. 1998. Development of a new microsporidian parasite, *Intrapredatorus barri* n. g., n. sp. (Microsporida: Amblyosporidae) from the predacious mosquito *Culex fuscanus* Wiedman (Diptera: Culicidae). *Parasitol. Inter.,* **47**:183–193.

Flegel, T. W. & Pasharawipes, T. 1995. A proposal for typical eukaryotic meiosis in microsporidians. *Can. J. Microbiol.,* **41**:1–11.

Hazard, E. I. & Oldacre, S. W. 1975. Revision of microsporidia (Protozoa) close to *Thelohania* with descriptions of one new family, eight new genera, and thirteen new species. *U. S. Dept. Agric. Tech. Bull.,* **1530**:1–104.

Hazard, E. I., Fukuda, T. & Becnel, J. J. 1985. Gametogenesis and plasmogomy in certain species of Microspora. *J. Invertebr. Pathol.,* **46**:63–69.

Johnson, M. A., Becnel, J. J. & Undeen, A. H. 1997. A new sporulation sequence in *Edhazardia aedis* (Microsporidia: Culicosporidae), a parasite of the mosquito *Aedes aegypti* (Diptera: Culicidae). *J. Invertebr. Pathol.,* **70**:69–75.

Larsson, J. I. R. 1989. On the ultrastructure of *Hyalinocysta explitoria* (Microspora, Thelohaniidae) and the taxonomy of the species. *J. Invertebr. Pathol.,* **54**:213–223.

Larsson, R. 1983. Description of *Hyalinocysta explitoria* n. sp., a microsporidian parasite of the blackfly *Odagmia ornata*. *J. Invertebr. Pathol.,* **42**:348–356.

Loubès, C. 1979. Recherches sur la méiose chez les microsporidies: conséquences sur les cycles biologiques. *J. Protozool.* **26**:200–208.

Lucarotti, C. J. & Andreadis, T. G. 1995. Reproductive strategies and adaptations for survival among obligatory microsporidian and fungal parasites of mosquitoes: a comparative analysis of *Coelomomyces* and *Amblyospora*. *J. Am. Mosq. Control Assoc.,* **11**:111–121.

Micieli, M. V., Garcia, J. J. & Becnel, J. J. 1998. Horizontal transmission of *Amblyospora dolosi* (Microsporidia: Amblyosporidae) to the copepod *Metacyclops mendocinus* (Wierzejski, 1892). *J. Invertebr. Pathol.,* **72**:330–335.

Micieli, M. V., Garcia, J. J. & Becnel, J. J. 2000a. Life cycle and description of *Amblyospora camposi* n. sp. (Microsporidia: Amblyosporidae) in the mosquito *Culex renatoi* (Diptera: Culicidae) and the copepod *Paracyclops fimbriatus fimbriatus* (Copepoda: Cyclopidae). *J. Eukaryot. Microbiol.,* **47**:575–580.

Micieli, M. V., Garcia, J. J. & Becnel, J. J. 2000b. Horizontal transmission of *Amblyospora albifasciati* Garcia and Becnel, 1994 (Microsporidia: Amblyosporidae) to a copepod intermediate host and the neotropical mosquito, *Aedes albifasciatus* (Macquart, 1837). *J. Invertebr. Pathol.,* **75**:76–83.

Sweeney, A. W., Doggett, S. L. & Piper, R. G. 1990. Life cycle of *Amblyospora indicola* (Microspora: Amblyosporidae), a parasite of the mosquito *Culex sitiens* and of *Apocyclops* sp. copepods. *J. Invertebr. Pathol.,* **55**:428–434.

Sweeney, A. W., Doggett, S. L. & Piper, R. G. 1993. Life cycle of new species of *Duboscqia* (Microsporidia: Thelohaniidae) infecting the mosquito *Anopheles hilli* and an intermediate copepod host, *Apocyclops dengizicus*. *J. Invertebr. Pathol.,* **62**:137–146.

Sweeney, A. W., Graham, M. F. & Hazard, E. I. 1988. Life cycle of *Amblyospora dyxenoides* sp. nov. in the mosquito, *Culex annulirostris* and the copepod *Mesocyclops albicans*. *J. Invertebr. Pathol.,* **51**: 46–57.

Sweeney, A. W., Hazard, E. I. & Graham, M. F. 1985. Intermediate host for an *Amblyospora* sp. (Microspora) infecting the mosquito, *Culex annulirostris*. *J. Invertebr. Pathol.,* **46**:98–102.

Swofford, D. 1993. PAUP User's Manuel. Version 3.1.1. Illinois Natural History Survey, Champaign-Urbana, Illinois.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.,* **22**:4673–80.

Vavra, J., Bai, M. G. & Panicker, K. N. 1984. *Amblyospora indicola* sp. n., a microsporidian pathogen of the mosquito *Culex sitiens*. *Folia Parasit. (Praha),* **31**:207–213.

Vossbrinck, C. R., Andreadis, T. G. & Debrunner-Vossbrinck, B. A. 1998. Verification of intermediate hosts in the life cycles of Microsporidia by small subunit rDNA sequencing. *J. Eukaryot. Microbiol.,* **45**:290–292.

Weiss, L. M. & Vossbrinck, C. R. 1999. Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. *In*: Wittner, M. & Weiss, L. M. (ed.), The Microsporidia and Microsporidiosis. American Society for Microbiology Press, Washington. **4**:129–171.

White, S. E., Fukuda, T. & Undeen, A. H. 1994. Horizontal transmission of *Amblyospora opacita* (Microspora: Amblyosporidae) between the mosquito, *Culex territans*, and the copepod, *Paracyclops fimbriatus chiltoni*. *J. Invertebr. Pathol.,* **63**:19–25.

Received 02/21/02, 05/21/02; accepted 05/21/02