

Amblyospora trinus N. Sp. (Microsporida: Amblyosporidae) in the Australian Mosquito *Culex halifaxi* (Diptera: Culicidae)

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ABSTRACT. A new species of *Amblyospora*, a parasite found in wild populations of the predacious Australian mosquito *Culex halifaxi*, was investigated with light and electron microscopy. This species was found to be heterosporous with two concurrent sporulation sequences in the host larvae, both arising from diplokaryotic meronts and ending with haploid spores. One sequence was dominant and involved meiosis to produce eight thick-walled, broadly oval meiospores in a sporophorous vesicle (SV). The other sequence involved nuclear dissociation to produce lanceolate, thin-walled spores in a subpersistent SV. Horizontal transmission to the mosquito host, by one or both of two distinctly different pathways (one via an intermediate host, the other by cannibalism of infected individuals) and by vertical transmission, are postulated but have not been demonstrated. A new species, *Amblyospora trinus*, is proposed and its affinities to other heterosporous microsporidia in mosquitoes are discussed.

Key words. *Amblyospora trinus* n. sp., life cycle, Microsporida, taxonomy, ultrastructure.

DURING a 1983 survey of Australian mosquitoes for pathogenic microorganisms, several larvae of the predacious mosquito *Culex halifaxi* Theobald, 1903, were found infected with a microsporidium. Our initial observations revealed the presence of eight oval spores within a sporophorous vesicle (SV), implicating this species as a member of the genus *Amblyospora* Hazard & Oldacre, 1975. Subsequent investigation revealed the presence of a 2nd concurrent sporulation sequence, a unique feature for an *Amblyospora* sp. [3]. This study describes the developmental sequences and morphological features of this new and unusual microsporidium. A new species is proposed and its affinities to other heterosporous microsporidia in mosquitoes are discussed.

MATERIALS AND METHODS

Larvae of *C. halifaxi* were collected in March 1983 from a shaded rain forest stream (clear, slow-flowing water) at the foot of the Brown Range near Cowley Beach, Queensland (Australia). Six of about 40 larvae had patent fat-body infections of a microsporidium; this material was used in this study. Giemsa and Heidenhain-stained material were prepared according to previously described protocols [10]. Adipose tissues for electron microscopy (EM) were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), washed in buffer, and held in the cold until returning to the laboratory. Larval tissues were postfixed in 1% Dalton's chrome osmium in the cold for 3 h and the osmium was replaced with 0.5% aqueous uranyl acetate for 1 h. These tissues were dehydrated through an ascending ethanol and acetone series and embedded in Epon-Araldite. Gold thin sections were poststained with methanolic uranyl acetate followed by lead citrate.

RESULTS

This species proved to be heterosporous with two concurrent sporulation sequences that occurred in all six larvae examined. Both sequences were initiated by transformation of diplokaryotic meronts into sporonts; these diplokaryotic cells underwent haplosis by either meiosis or nuclear dissociation. Subsequent sporulation ended with two different types of haploid spores. Evidence for the occurrence of an additional developmental sequence in the adult female, theoretically ending with the binucleate spores typical of *Amblyospora*, was observed.

Meronts and merogony. Meronts were round or oval diplokaryotic cells (Fig. 1, 11, 30). Merogony apparently was limited to binary division (Fig. 2). During merogony, cytokinesis was delayed with the production of temporary plasmodia containing two pairs of nuclei; plasmodia with more nuclei were not observed. Meronts proliferated in large numbers in the hyaloplasm

of adipose cells. The final products of merogony transformed into two kinds of sporonts, the initial stages of the sporulation sequences.

Sporulation sequence involving meiosis. Fusiform stages that we interpret as transitional forms were found in both Giemsa and EM preparations (Fig. 3, 12). Although these sometimes appeared uninucleate in Giemsa-stained material indicating karyogamy, only diplokaryotic stages were observed with the electron microscope. Clumps of dispersed chromatin occurred in the nuclei with spindle plaques commonly observed on the envelope. The cytoplasm contained small arrays of endoplasmic reticulum and free ribosomes (Fig. 12). The fusiform cells apparently transformed directly into diplokaryotic sporonts.

In EM sections, the sporont was recognizable first by a thickened (doubled) plasmalemma and had an extensive Golgi reticulum throughout the cytoplasm (Fig. 13). The small vesicles of the Golgi were clustered tightly and sometimes closely associated with the nuclear envelope. Rough endoplasmic reticulum was well developed in the cytoplasm of these sporonts (Fig. 13, 14, 15).

In Giemsa-stained material, sporonts with nuclei entering meiosis were identifiable by a halo that represented the envelope of the future SV (Fig. 4). The two nuclei of the diplokaryon lost their attraction for one another and the chromatin became less dispersed (Fig. 13). As the process proceeded, the envelope of the future SV separated from the plasmalemma and dense masses of secretory granular material accumulated in the episporontal space (Fig. 14, 15). A suspected spindle plaque (similar to the one observed in Fig. 45 of Ref. 5) was located between the two members of the diplokaryon with the two nuclei only loosely associated (Fig. 15). Synaptonemal complexes were present in each of these paired nuclei, indicating that the nuclei were in the zygotene or pachytene stage of prophase I. Unidentified membrane-bound vacuoles with electron-dense inclusions were observed in both the nucleus and cytoplasm. The cytoplasm contained the Golgi reticulum and numerous arrays of rough endoplasmic reticulum (Fig. 15).

Our observations of meiotic divisions were incomplete. Stages observed after prophase I were metaphase I (Fig. 5), telophase I (Fig. 6, 17), and final stages of meiosis II (Fig. 7, perhaps Fig. 16, 18). A 3rd equatorial division resulted in eight nuclei (Fig. 8). Cytokinesis was incomplete until the end of the 3rd nuclear division; then it went to completion producing eight uninucleate sporoblasts.

Secretory tubular material produced during sporogony, usually appeared concentrated on one side of the sporogonial plasmodium (Fig. 14-17). In one instance, the tubules were attached to the particular part of the plasmodium that was situated near-

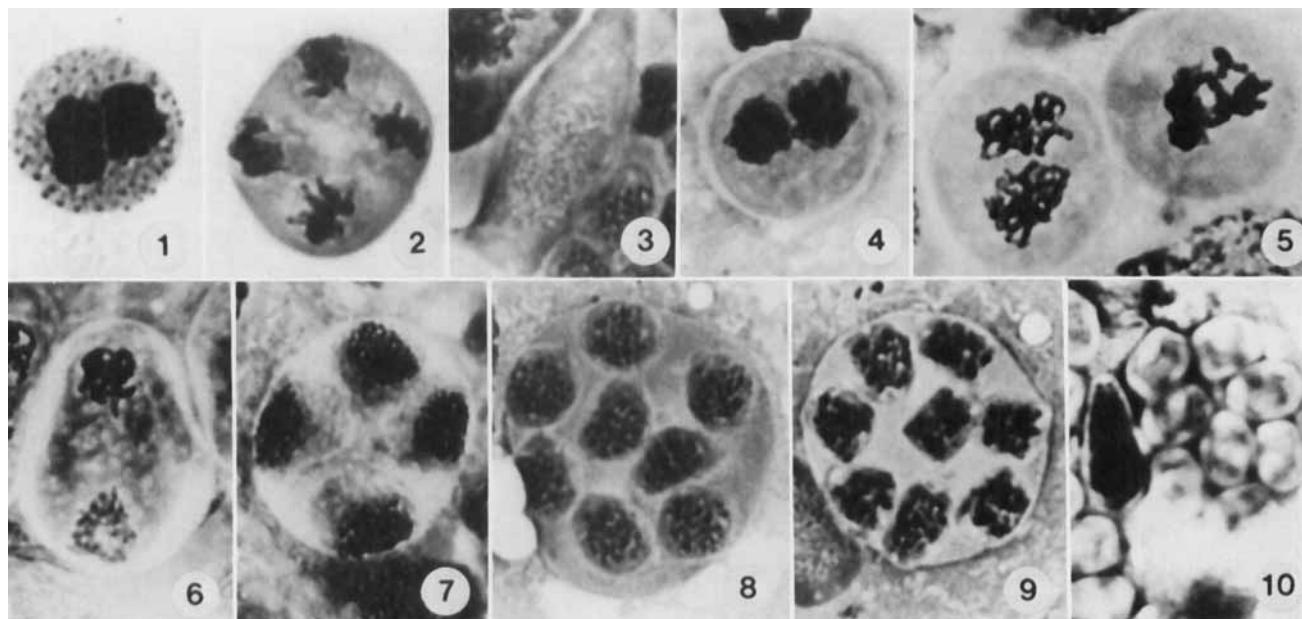


Fig. 1-10. Sporulation involving meiosis. Photomicrographs of Giemsa-stained material at $\times 2,000$. 1. Diplokaryotic meront. 2. Diplokaryotic stage dividing. 3. Fusiform stage with what appears to be a large, single nucleus (synkaryon?). 4. A sporont with the envelope of the future SV indistinctly evident as a halo around the cell. 5. Slightly more advanced sporonts (left with chromosomes of each member of the diplokaryon condensed, right at metaphase with a single nucleus due to the coalescence of the two members of the diplokaryon). 6. Binucleate stage after the 1st meiotic division. 7. Tetrnucleate sporogonial plasmodium after the 2nd meiotic division. 8. Octonucleate sporogonial plasmodium. 9. Eight sporoblasts within the future SV (Note the pyriform spore formed after nuclear dissociation).

est the mass of episporontal material, indicating that secretion was probably a function of a specialized area of the cytoplasm (Fig. 16). At the end of sporogony, the secretory products among the sporoblasts appeared to organize into sheets which were often in a close, parallel relationship with the developing spore wall of sporoblasts (Fig. 19). As sporogenesis proceeded, the material in the episporontal space disappeared (Fig. 9, 20, 21).

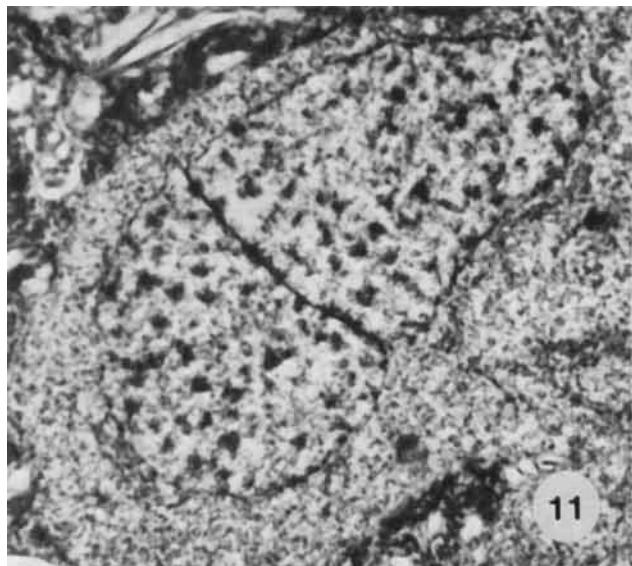
Eight meiospores enclosed in the SV, characteristic of *Amblyospora* [8], were the end products of sporulation (Fig. 10, 21, 22). These spores were barrel-shaped in Giemsa-stained preparations (Fig. 10) and ultrastructurally were broadly oval and thick-walled (Fig. 22). The exospore had the structure of Larsen's Type IIIC [14], characteristic of the species *Amblyospora*. The exospore was laminated with an outer double layer that resembled a unit membrane and an inner layer that was thick and fibrous in appearance and absent at the anterior pole (Fig. 22, 23). Meiospores had a lamellate polaroplast with an anisofilar polar filament that made eight turns about the posterior vacuole; spores measured $4.30 \pm 0.04 \times 3.44 \pm 0.03 \mu\text{m}$ (mean \pm SE) in Heidenhain-stained material.

Sporulation sequence involving nuclear dissociation. Sporulation began when the final products of merogony, recognizable in Giemsa-stained preparations by dark-staining cytoplasm and compact nuclei, underwent nuclear dissociation (Fig. 24-26). Nuclear dissociation was followed by cytokinesis (Fig. 27, 31) that resulted in two uninucleate sporoblasts (Fig. 28, 33). Alternatively, perhaps, this 1st generation of uninucleate cells did not directly transform into spores but divided into sporoblasts. In this case, they would be considered sporonts and the prior cells that underwent nuclear dissociation, sporont mother cells. Sporogonial plasmodia were not observed.

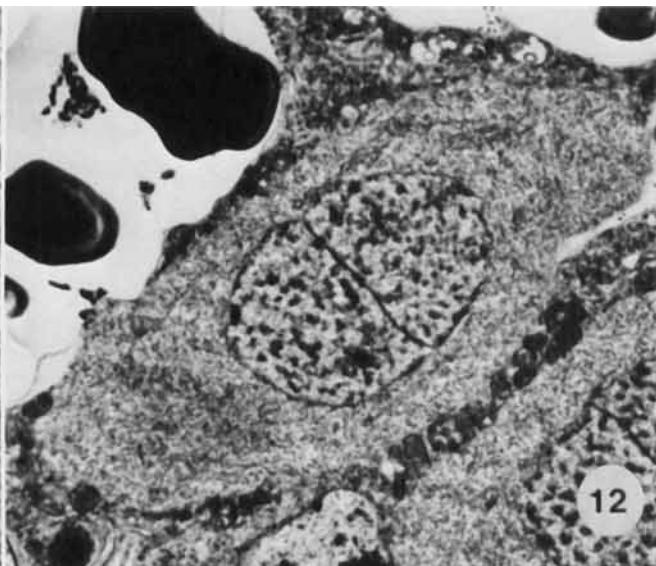
In EM sections, the dividing sporont was distinguished by the formation of a delicate membrane (the envelope of the future SV) on the plasmalemma within which amorphous secretory material accumulated (Fig. 31, 32). Cytoplasmic changes were also apparent by the increase of both the endoplasmic reticulum and components of the Golgi system (Fig. 31). The delicate membrane, subsistent as a SV, enveloped the sporoblasts along with an accumulation of amorphous material (Fig. 32-34). The polar filament was formed by the Golgi apparatus of

Fig. 11-16. Electron micrographs of early stages in the sporulation sequence involving meiosis. 11. Diplokaryotic meront. $\times 9,400$. 12. Fusiform diplokaryotic stage prior to the beginning of meiosis. $\times 6,400$. 13. Sporont, primordium of the envelope of the future SV evident as thickening (arrow) and blisters on the plasmalemma. $\times 6,000$. 14. Sporont with granular material within the envelope of the future SV (ESV). Golgi (G) ($\times 6,000$). 15. Enlargement of Fig. 14, synaptosomal complexes (SC) with a suspected spindle plaque (SP) located between the two members of the diplokaryon. Arrows indicate vacuoles with dense inclusions in the nucleus and the cytoplasm. Rough endoplasmic reticulum (RER). $\times 10,000$. 16. Sporogonial plasmodium with tubular secretory materials attached to one side (P). Envelope of the future SV (ESV). $\times 6,500$.

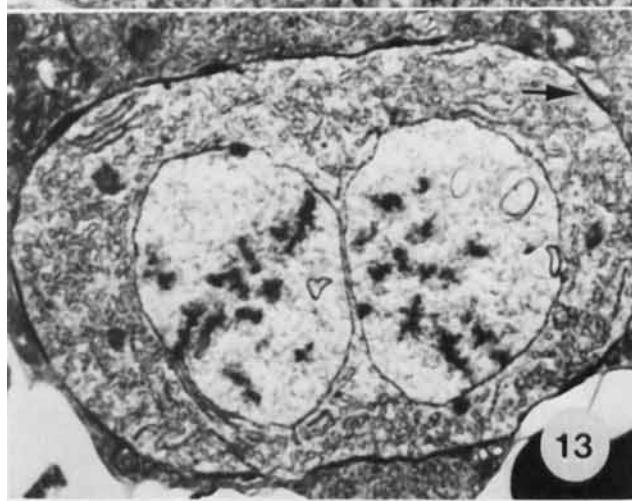
Fig. 17-23. Electron micrographs of later stages in sporulation involving meiosis. 17. Binucleate stage within the future SV. $\times 6,000$. 18. Tetrnucleate sporogonial plasmodium, cytokinesis incomplete. $\times 9,600$. 19. Sporoblasts within the future SV, granular material appears sheet-like (arrow). $\times 6,000$. 20. Four sporoblasts. $\times 6,500$. 21. Three immature spores, granular material absent. $\times 10,400$. 22. Mature meiospore. $\times 14,400$. 23. Spore wall, endospore (EN) and exospore (EX). $\times 96,000$.



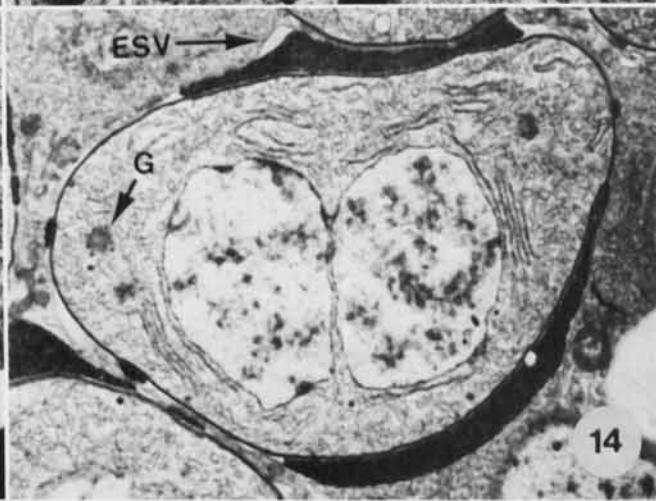
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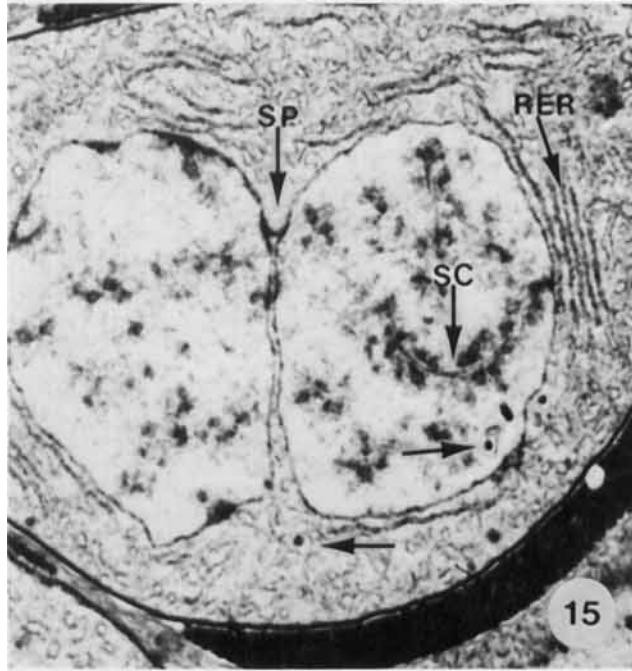
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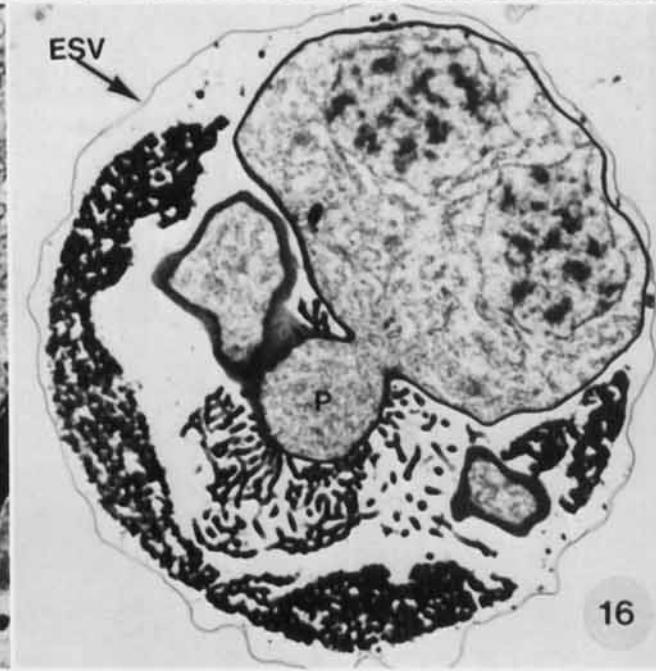
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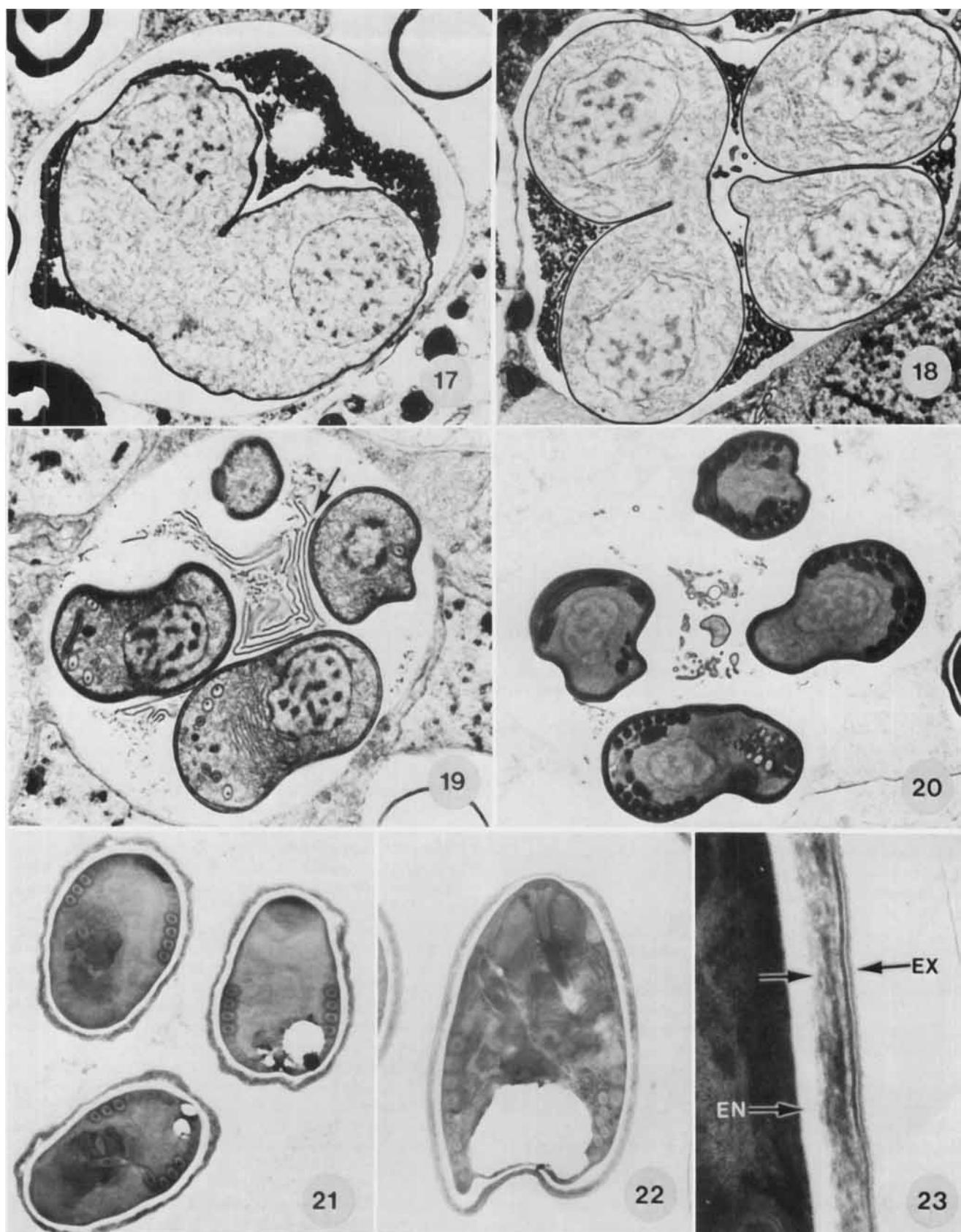
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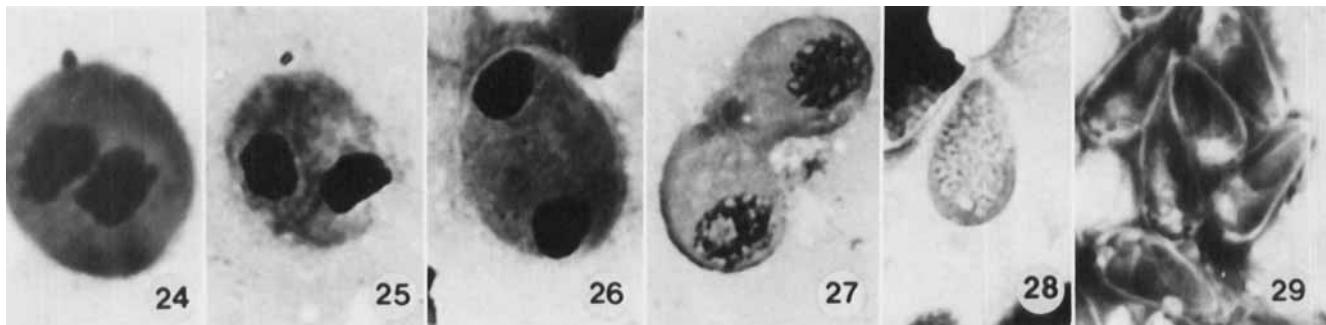


Fig. 24-29. Sporulation involving nuclear dissociation. Photomicrographs of Giemsa-stained material at $\times 2,000$. 24. Diplokaryotic sporont with nuclei loosely associated. 25. Nuclear dissociation. 26. Sporont after nuclear dissociation with the two nuclei at opposite ends of the cell. 27. Sporont undergoing cytokinesis. 28. Uninucleate sporoblast. 29. Uninucleate pyriform spores.

the sporoblast, usually preceding polaroplast formation (Fig. 34, 35).

The uninucleate spores, produced after nuclear dissociation, possessed an extensive polaroplast that occupied the anterior two-thirds of the spore (Fig. 36, 38). The polaroplast was divided into chambers by a helical arrangement of septa (Fig. 36), probably derived from the "filament cytoplasmic sheath" (Fig. 37) as described by Vávra [20]. Mature uninucleate spores (usually one or two contained within a delicate SV) were lanceolate and measured $8.47 \pm 0.12 \times 3.72 \pm 0.05 \mu$ in Heidenhain-stained material (Fig. 29). The polar filament made 12–13 turns in the posterior region of these thin-walled, uninucleate spores (Fig. 38). The exospore was thin and without lamination (Fig. 39).

Evidence supporting the occurrence of an additional developmental sequence. Uninucleate stages, characteristic of early stages in the horizontally induced sequence in *Edhazardia aedis* (Kudo, 1930) Becnel, Sprague, & Fukuda, 1989 [5]; *Culicospora magna* (Kudo, 1920) Weiser, 1977 [4]; and *Amblyospora* spp. [2, 18] that ended with binucleate spores, were found in a Giemsa-stained preparation of an adult female *C. halifaxi*. Diplokaryotic stages or binucleate spores were not observed.

DISCUSSION

Some Aspects of Comparative Development

Sporulation involving meiosis. The sporulation sequence of this species that involved meiosis was similar to those reported for various *Amblyospora* spp. without significant variations [2, 6, 9, 18]. Fusiform diplokaryotic stages, characteristic of cells entering karyogamy, preceded sporont formation and the sporulation sequence that ended with eight meiospores within a SV [4, 6]. The morphology of the meiospores of this species is essentially similar to that reported for other species of *Amblyospora* [8, 15].

In previous studies, this sporulation sequence occurred in larvae infected transovarially and was usually related to the sex of the host, causing mortality in male progeny [12, 13]. The origin of larval infections in *C. halifaxi* is uncertain. In most other species [2, 4, 5, 18], infections in the adult host are initiated by horizontal transmission to mosquito larvae wherein uninucleate stages (gametes) undergo a developmental sequence that involves plasmogamy and nuclear association to form diplokaryotic cells. Transformation of these cells gives rise to binucleate spores in the female adult that are responsible for transovarial transmission. Based on our observations of early uninucleate stages in the adult female of *C. halifaxi*, we postulate that this species is probably transmitted transovarially as in other previously studied species of microsporidia in mosquitoes.

Sporulation involving nuclear dissociation. The sporulation sequence that involved nuclear dissociation for this species was similar in most respects to that reported for *C. magna* [4] and *E. aedis* [5]. *Amblyospora trinus* differed because sporogony was by binary division, whereas in the other two species it was commonly multiple in addition to binary division.

The morphology of the uninucleate spores formed after nuclear dissociation is characteristic of that found in *E. aedis* [5], *Culicospora magna* [4], and *Amblyospora* spp. [1, 18]. In each of these species, the uninucleate spores of this type are distinguished by a large and compartmentalized polaroplast of similar origin and form. Another common feature is that usually each spore is individually contained within a delicate SV.

In *Amblyospora dyxenoides* Sweeney, Graham & Hazard, 1988, this type of uninucleate spore is formed in the copepod intermediate host *Mesocyclops albicans* [19] but in *Amblyospora connecticus* Andreadis, 1988, it is formed in the copepod *Acanthocyclops vernalis* [1]. The copepod intermediate hosts in these species are infected orally by meiospores formed in transovarially infected larval mosquitoes. The resulting uninucleate spores are infectious per os to mosquito larvae.

Culicospora magna has been shown to form a spore virtually identical to the uninucleate lanceolate spores formed in *C. halifaxi* and some *Amblyospora* spp. In *C. magna*, this spore is formed after nuclear dissociation in the mosquito host following transovarial transmission [4]. Uninucleate spores are directly responsible for horizontal transmission (per os) to the mosquito host without the involvement of an intermediate host.

Sporulation without haploidy. Although information on the complete developmental cycle of *A. trinus* is unavailable, observations of uninucleate parasite stages in an adult suggest the involvement of a 3rd sporulation sequence. These stages are consistent with developmental sequences previously observed in other adult female mosquitoes, the end product of which is binucleate spores responsible for transovarial transmission.

Sporulation in intermediate hosts. It is possible that meiospores of *A. trinus* initiate a horizontally transmitted developmental sequence in an intermediate host, perhaps a copepod, that ends with spores infectious to the mosquito host. This latter pathway (a possible 4th sporulation sequence) would be representative of those known for several species of *Amblyospora* [2, 19].

Concurrence of sporulation sequences. The presence of concurrent sporulation sequences in the present species is not unique in heterosporous microsporidia of mosquitoes although it may be unique for species of *Amblyospora*. *Hazardia milleri* (Hazard & Fukuda, 1974) Weiser, 1977 forms two spore types simul-

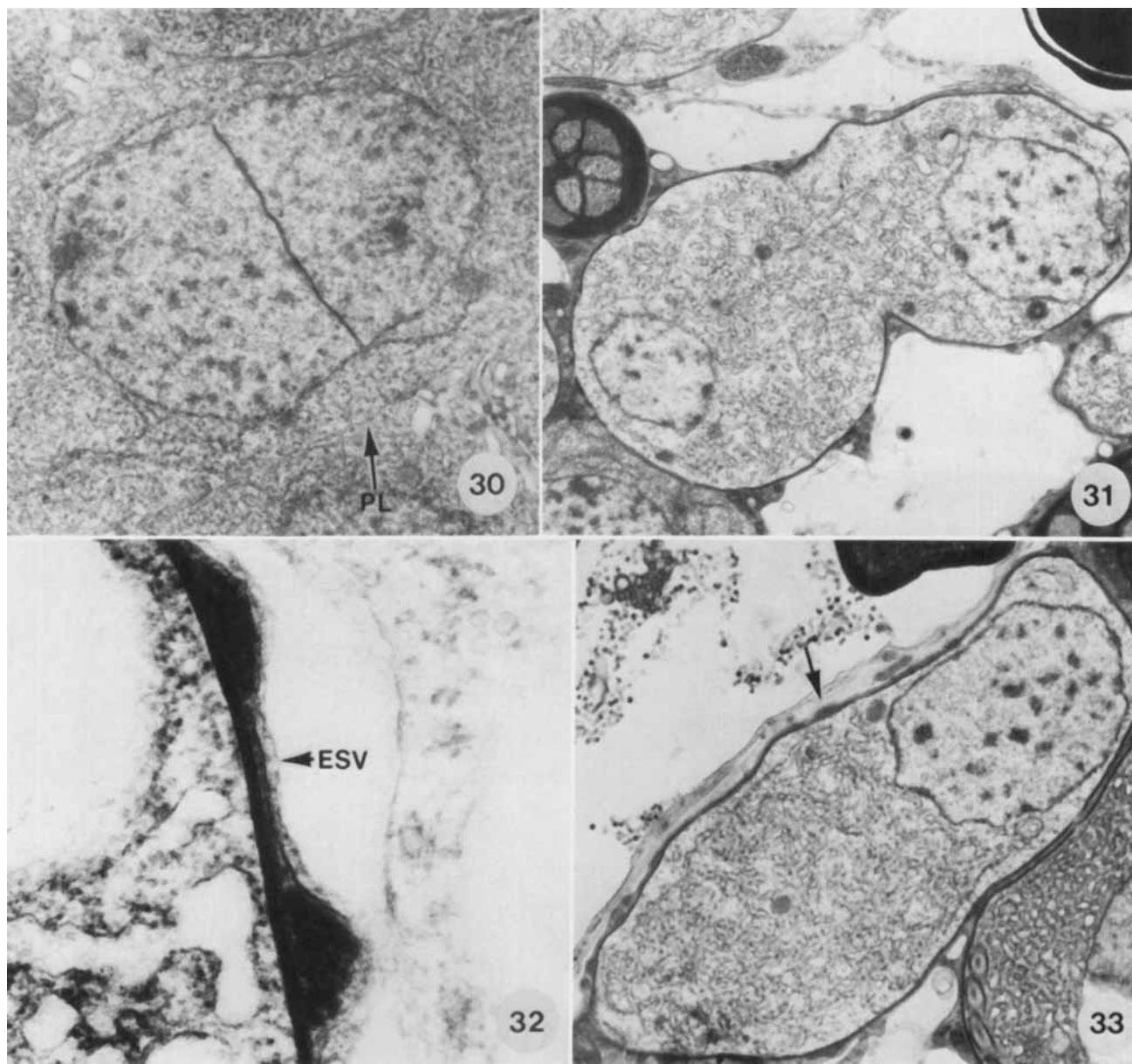
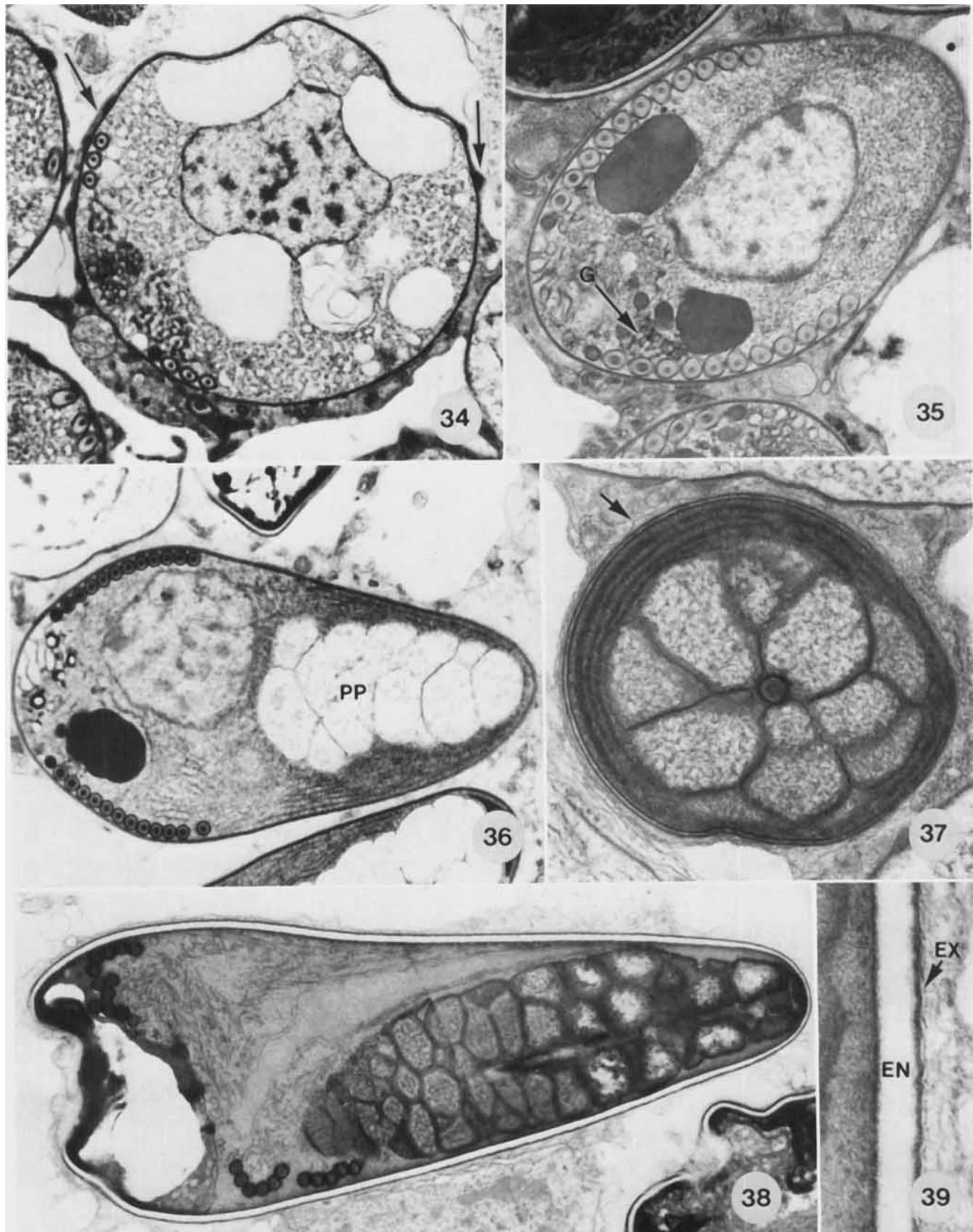


Fig. 30-33. Electron micrographs of early stages in sporulation involving nuclear dissociation. 30. Diplokaryotic meront limited by delicate plasmalemma (PL). $\times 14,000$. 31. Sporont dividing. $\times 9,600$. 32. Enlargement of the plasmalemma demonstrating the elaboration of the envelope of the future SV (ESV). $\times 61,000$. 33. Early sporoblast, arrow indicating amorphous secretory materials within the future SV. $\times 14,000$.

taneously in larval adipose tissue of *Culex quinquefasciatus* [7]. In the predominant sporulation sequence, diplokaryotic sporonts give rise to sporogonial plasmodia producing uninucleate spores without the involvement of meiosis. In the 2nd less frequent sequence, diplokaryotic sporonts divide directly into sporoblasts that transform directly into thick-walled binucleate spores.

Two other species in mosquitoes have concurrent sporulation sequences but in both cases only one spore type forms successfully because the sequence involving meiosis aborts. The successful sporulation sequence in *Culicosporella lunata* (Hazard & Savage, 1970) Weiser, 1977 involves diplokaryotic sporogonial plasmodia producing binucleate spores [11]. In *E. aedis*, uninucleate spores are produced by a 2nd sporulation sequence involving nuclear dissociation and sporogonial plasmodia [5].

It is uncertain whether concurrent sporulation sequences in an *Amblyospora* species have been reported. Simmers reported the occurrence of *Amblyospora opacita* (Kudo, 1922) Hazard & Oldacre, 1975 infections in *Culiseta inornata* larvae with concurrent infections of *Culicospora magna* in two of them [17]. In the same collection, *C. restuans* and *Anopheles punctipennis* larvae were reportedly infected with only *C. magna* and some *C. inornata* larvae with only *A. opacita*. Becnel horizontally transmitted *C. magna* to *C. restuans* but not to other mosquitoes tested, including *C. inornata* and *A. quadrimaculatus*, suggesting a very restrictive host range for *C. magna* [4]. Because the uninucleate spores of *C. magna* are virtually identical to the ones formed in *Amblyospora* spp., Simmers actually may have observed concurrent sporulation sequences of one *Amblyospora* sp. and not concurrent infections by two species. Clearly, trans-



mission studies are required to resolve whether the Simmers' report represents dual infections of *A. opacita* and *C. magna* or an *Amblyospora* species that expresses two spore types simultaneously as found in the present species.

Origin of sporophorous vesicles (SV). While the evidence presented here indicates that the "vesicle" found during sporulation involving nuclear dissociation is of parasite origin, the exact stage (either sporont or sporoblast) giving rise to this structure is debatable. The difficulty is in distinguishing an early sporoblast from a sporont. We have determined that in this species, the sporont gave rise to the future SV.

In *A. connecticus*, sporoblasts reportedly secrete a subsistent SV during the sporulation sequence in the copepod intermediate host *A. vernalis* [1, 2]. For *A. dyxenoides* in *M. albicans*, the particular stage that elaborates this structure during the sporulation sequence is not given. The result, however, is similar in that spores are individually contained within a SV [18, 19]. In the intermediate host *Macrocylops albidus*, sporonts of *Amblyospora californica* gave rise to the vesicle (unpubl.). In *C. magna* and *E. aedis*, the SV is initiated during sporogony apparently by the sporont [4, 5].

Larsson recently suggested that the vesicle containing uninucleate spores of some *Amblyospora* spp. formed in the copepod intermediate host is not legitimately a SV [16]. Larsson reports this vesicle is a "sac, produced by delamination of exospore material from the sporoblast, not a SV produced by the sporont." The evidence does not support the suggestion that material from the exospore is involved in this process. In the present species and the species discussed above, the SV appears to form as a doubling of the plasmalemma [1, 4, 5]. We have no difficulty referring to this (or any structure holding spores) as the SV, regardless of whether sporonts or sporoblasts are involved in formation.

The presence or absence of the SV is an important taxonomic character for the microsporidia. To describe a species as lacking a SV because the vesicle is initiated by the sporoblast rather than the sporont (often subjective) could cause more taxonomic problems than it could resolve.

Transmission. In previously studied species, infections in the adult mosquito are acquired when larvae ingest uninucleate spores formed in one of two ways. In some species, the lanceolate, uninucleate spores are formed in larval mosquitoes after dissociation of the diplokaryon [4, 5]. In other species, lanceolate, uninucleate spores may be formed in a copepod intermediate host after ingestion of meiospores [2, 19]. Based on our findings, the present species may invade the mosquito host by both of these pathways.

Culex halifaxi is an unusual mosquito that is both predacious and cannibalistic. Cannibalism of infected larvae would provide the opportunity for the uninucleate spores formed after nuclear dissociation to initiate a horizontal transmission sequence in the mosquito similar to those in *C. magna* and *E. aedis* [4, 5]. In addition, as already discussed, meiospores may initiate a developmental sequence in a copepod intermediate host similar to those in *Amblyospora* spp. [2, 19]. Regardless of the path, once the parasite enters the mosquito host, a developmental

sequence that involves plasmogamy and nuclear association that ends with binucleate spores, is predicted. Binucleate spores would transovarially transmit the pathogen as occurs in other heterosporous microsporidia of mosquitoes.

Classification. The present species has affinities for the type species of three established genera based on morphology and development (*Amblyospora*, *Culicospora*, and *Edhazardia*). However, certain important aspects of the developmental cycle are incompletely known, particularly the sequence in the adult suspected of involving transovarial transmission and the potential role of an intermediate host. Until additional information on the developmental cycle is obtained that would dictate a change, *A. trinus* is retained in the genus *Amblyospora*.

SYSTEMATICS

Amblyospora trinus n. sp.

Amblyospora sp. Sweeney in Beclen, 1986, *Proceed. Fourth Intern. Coll. Invertebr. Pathol.*, p. 333.

Type host. *Culex halifaxi* Theobald, 1903 (Diptera: Culicidae).

Site of infection. Fat body of larvae. Undetermined in the adult.

Parasite-host cell relations. Parasites in hyaloplasm of fat cell during the observed presporulation stages. Sporulation in vesicles during the two observed sequences.

Development. Merogony by binary division. With two concurrent sporulation sequences, both arising from diplokaryotic meronts and ending with haploid spores. One sporulation sequence dominant, involving meiosis and ending with eight meiospores within a SV. The other sequence disporous, involving nuclear dissociation and production of uninucleate, lanceolate spores each within a delicate SV.

Spore morphology. Both spore types uninucleate. Meiospores broadly oval, measuring $4.30 \pm 0.04 \times 3.44 \pm 0.03 \mu$ (Heidenhain-stain). Exospore laminated, the inner layer thick and fibrous. Polar filament anisofilar, singly coiled with eight turns about the posterior vacuole. Lanceolate, thin-walled spores $8.47 \pm 0.12 \times 3.72 \pm 0.05 \mu$ (Heidenhain-stain). Polar filament isofilar with 12–13 turns in the posterior of the spore. Polaroplast lamellate, divided into chambers by a helical arrangement of septa, occupying the anterior two thirds of the spore.

Type locality. Brown Range adjacent to Cowley Beach, Queensland, Australia. Latitude: 17° 41' S; longitude 146° 07' E.

Transmission. Transovarial and Per os (by inference).

Specimens deposited. Type slides (holotype plus three paratypes) have been deposited at the International Protozoan Type Slide Collection, Smithsonian Institution (USNM Nos. 42353–56).

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Fig. 34–39. Electron micrographs of later stages in sporulation involving nuclear dissociation. 34. Uninucleate sporoblast, the envelope of the future SV (arrows) still closely applied to the plasmalemma within which are amorphous materials. $\times 10,900$. 35. Early sporogenesis, Golgi (G) involved in polar filament formation. $\times 18,000$. 36. Later sporogenesis, the polaroplast (PP) vacuolated and compartmentalized. $\times 12,200$. 37. Cross section of developing polaroplast. Note compartments of the polaroplast are limited by membranes apparently continuous with the filament cytoplasmic sheath. Sporophorous vesicle (arrow). $\times 32,000$. 38. Uninucleate spore. $\times 17,600$. 39. Spore wall, endospore (EN), and exospore (EX). $\times 96,000$.

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