

Amblyospora sp. (Microspora, Amblyosporidae) Infecting Nerve Ganglia of *Culex pipiens* (Diptera, Culicidae) from Egypt

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A species of *Amblyospora*-infecting neurones of *Culex pipiens* is described. Diplokaryotic meronts, which divided by binary fission, were distinguished at the electron microscope level by their unthickened plasma membranes. Sporonts with an electron-dense surface coat gave rise to eight uninucleate sporoblasts within a sporophorous vesicle, cytoplasmic division occurring at the quadridinucleate or octonucleate stages. Indications that nuclear fusion and chromosome reorganization occurred in merogony and sporogony were obtained by light microscopy but meiosis was not detected at the ultrastructural level. Spores were typical of *Amblyospora*, being ovoid when fresh, truncate when stained, and having an exospore of two membranous layers subtended by a thick amorphous layer, an electron-lucent endospore, an anisofilar polar filament, and a polaroplast comprised of an anterior region of close-packed lamellae and a posterior region of expanded sacs. The metabolic products in the sporophorous vesicle took the form of large globules, small globules with electron-dense borders, and fine granules. These were depleted in mature sporophorous vesicles, though a surface layer of fine granules on the spores may have been derived from them. Many stages were degenerate and it is suggested that *C. pipiens* may be an accidental host in which the parasite could develop suboptimally in nervous tissue only. Infections in larvae hatched from eggs in the laboratory indicate that vertical transmission occurs. © 1991 Academic Press, Inc.

INTRODUCTION

Numerous microsporidia infecting mosquitoes have been documented from many countries (Castillo, 1980). *Vavraia culicis* (formerly *Pleistophora culicis*) and *Nosema algeriae* often produce disseminated infections involving most, if not all, body tissues (Hazard and Chapman, 1977). Other species of *Pleistophora* have more restricted sites of infection in mosquito hosts, e.g., *Pleistophora collessi* in ovary (Laird, 1959), and *Pleistophora chapmani* and *Pleistophora caecorum* in midgut and gastric caeca (Clark and Fukuda, 1971; Chapman and Kellen, 1967). The octosporous genera, including most species of *Amblyospora* and *Parathelohania* in their mosquito hosts, are restricted to oenocytes and adipose tissue of larvae (Hazard and Chapman, 1977) and have diplokaryotic stages and spores in oenocytes and ovary

of adult females, which serve to transmit infections between generations (Hazard and Oldacre, 1975). *Amblyospora culicis* in *Culex quinquefasciatus* is the only species of the genus in which adipose tissue is recorded as a primary site of infection and nervous tissue as a secondary site (Toguebaye and Marchand, 1985). The first record of insect nervous tissue as the primary site of infection for microsporidia is that of Carter (1976) who recorded an infection in *Tipula paludosa* larvae but gave no morphological details nor attempted an identification. In this paper an *Amblyospora* sp. is described from the nervous tissue of *Culex pipiens*. This appears to be only the second record of microsporidia restricted to the nervous tissue of insects and the first report of a microsporidium restricted to the nervous tissues of mosquitoes.

MATERIALS AND METHODS

C. pipiens larvae were collected by sieving water from a small pond from Meet

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Ghamees Village near Mansoura City, north of Cairo, Egypt, during the summer of 1987. Larvae were checked for infection by examination from the ventral side against dark field illumination using a dissecting binocular microscope. Infected larvae were reared to adults under natural photoperiod conditions in 0.15% NaCl in tap water with dry yeast and toasted crumbs as a source of food. Some egg rafts, collected from the pond, were allowed to hatch and these larvae were also reared to adults. Some of these also developed infections. Smears of larvae of all instars and of newly emerged adults of both sexes were air-dried, fixed for 2 min in 95% methanol and stained with 10% Giemsa solution, pH 7.2. For histological studies, whole infected larvae and adults were fixed in Carnoy's solution, dehydrated in an ascending series of ethanol solutions, cleared in xylene, and embedded in paraplast (mp 58°C). Sections were cut at 5–7 µm and stained with Ehrlich's haematoxylin and counter-stained with eosin. A thin film of fresh spores was prepared using paraffin oil and studied by phase-contrast microscope. Measurements of fresh and stained spores were made using an eye-piece micrometer at $\times 1000$. For electron microscopy, infected larvae were cut into pieces composed of two body segments each. These were fixed in 5% glutaraldehyde, postfixed in 2.5% OsO₄, dehydrated in an ascending series of acetone solutions, and embedded in Spurr's resin. Sections, stained with Reynold's lead citrate and uranyl acetate, were examined using a Phillips EM300 electron microscope at accelerating voltage of 60 or 80 kV.

RESULTS

Infected nerve ganglia of second and third instar larvae appeared swollen and milky white through the transparent body cuticle, but it was increasingly difficult to identify infections in aging fourth instar larvae when the cuticle became thick and opaque.

In sections, nerve ganglia were seen to be

composed of two layers, an outer layer containing neurone cell bodies and an inner layer containing the proximal parts of axons and the dendrites of the nerve cells, together constituting the neuropile. The parasite developed only in the neurone layer (Fig. 1). In young larvae, only abdominal nerve ganglia were infected but, in the later instars, the infection spread to thoracic ganglia and in adults of both sexes the brain (Fig. 2) and even the optic lobes were infected.

Light Microscopy

Developmental stages were present in second and third instar larvae. Stages of merogony were rounded, some with a single diplokaryon size range between $9 \pm 0.1 \times 8 \pm 0.1$ and $9.2 \pm 0.1 \times 8.4 \pm 0.2$ µm (Fig. 3) and others with dividing nuclei (Fig. 4) or with two diplokarya with size range from $9 \pm 0.2 \times 8 \pm 0.1$ to $9.2 \pm 0.1 \times 8.4 \pm 0.1$ µm (Fig. 5). Division was by repeated binary fission. Dissociation of the nuclear envelope and chromosome mingling (Fig. 6) preceded stages with larger diplokarya which appeared to continue dividing (Fig. 7); size range differs from $10 \pm 0.2 \times 9.5 \pm 0.1$ to $11.1 \pm 0.3 \times 10 \pm 0.1$ µm. Production of a sporophorous vesicle around stages (now sporonts) with large nuclei associated as a diplokaryon was followed by another chromosome mingling (Figs. 8,9), size range between $8 \pm 0.1 \times 7.2 \pm 0.2$ and $11.1 \pm 0.3 \times 8 \pm 0.1$ µm. Further sporogonic divisions first gave rise to binucleate sporonts, size range from $10 \pm 0.3 \times 9 \pm 0.2$ to $11.1 \pm 0.2 \times 10 \pm 0.1$ µm (Figs. 10,11), then to tetra- and octonucleate sporonts, size range from $10 \pm 0.4 \times 9 \pm 0.1$ to $11.8 \pm 0.3 \times 10.2 \pm 0.1$ µm (Figs. 12–14). Cytokinesis occurred either at the tetranucleate stage followed by a further nuclear division or at the octonucleate stage, finally giving rise to eight uninucleate sporoblasts (Figs. 15,16) which matured into spores within the sporophorous vesicle (Figs. 17,18). The spherical sporophorous vesicles were persistent and measured 14.2

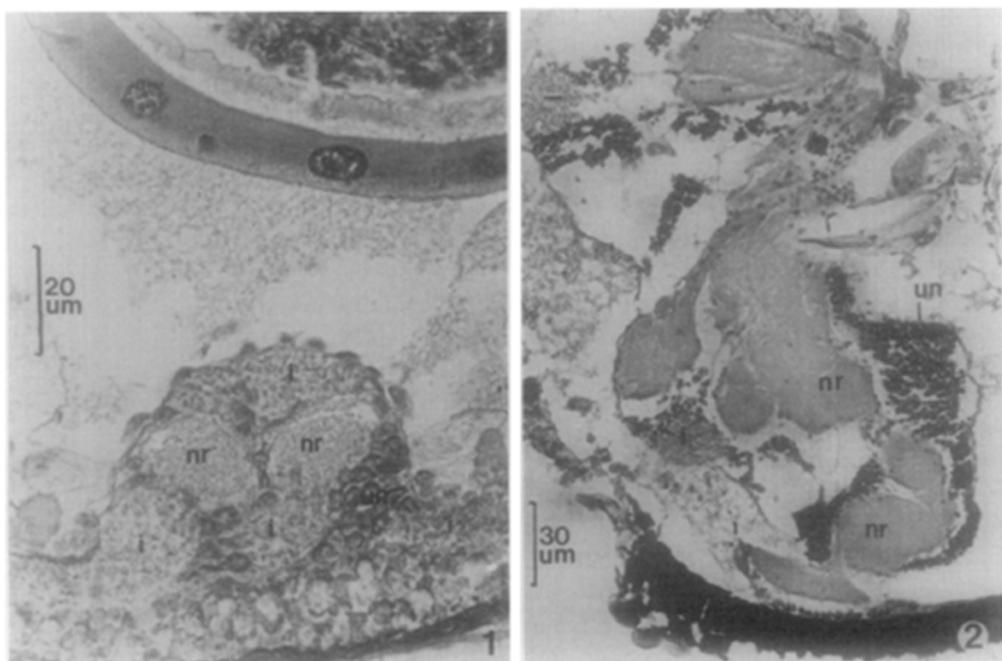


FIG. 1. Part of transverse section of *Culex pipiens* larva, showing nerve ganglion with uninfected neuropile (nr), microsporidian-infected neurones (i), and uninfected neurones (un).

FIG. 2. Longitudinal section of the brain of *Culex pipiens* adult illustrating uninfected neuropile (nr), microsporidian-infected neurones (i), and uninfected neurones (un).

$\pm 0.3 \times 12.4 \pm 0.1 \mu\text{m}$. Fresh spores measured $6.7 \pm 0.9 \times 4.2 \pm 0.6 \mu\text{m}$ and stained spores measured $5.6 \pm 0.8 \times 3.6 \pm 0.5 \mu\text{m}$ ($N = 50$).

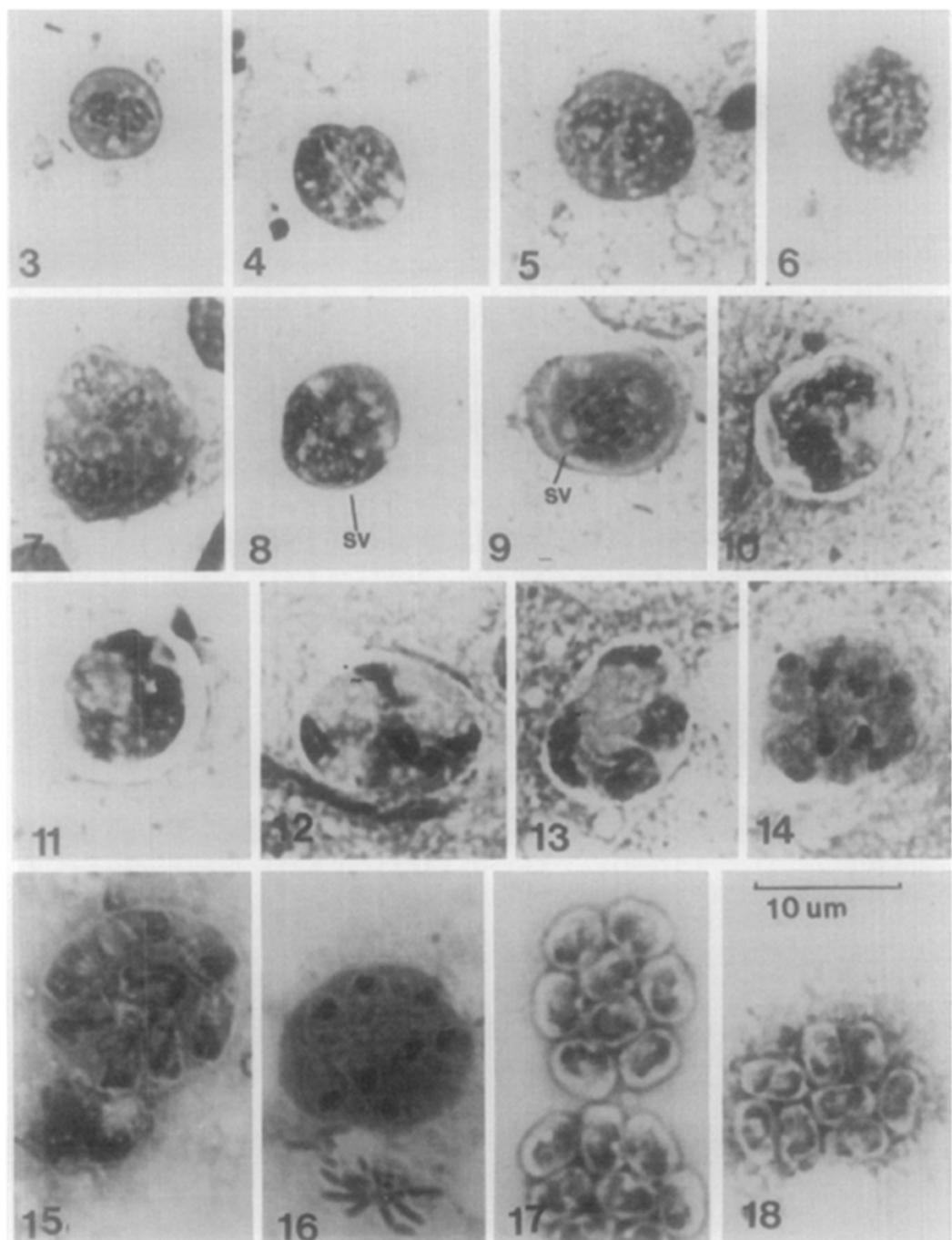
Electron Microscopy

All stages of development from meronts to mature octospores in sporophorous vesicles were enclosed by a single membrane, which suggested that there was multiple development within a single neurone (Fig. 19). This resulted in a complex series of membranes from which it was difficult to determine which were of host origin, unless residual cytoplasm with mitochondria was present. It was often difficult to differentiate the membranes of separate sporophorous vesicles containing parasites at different stages of development.

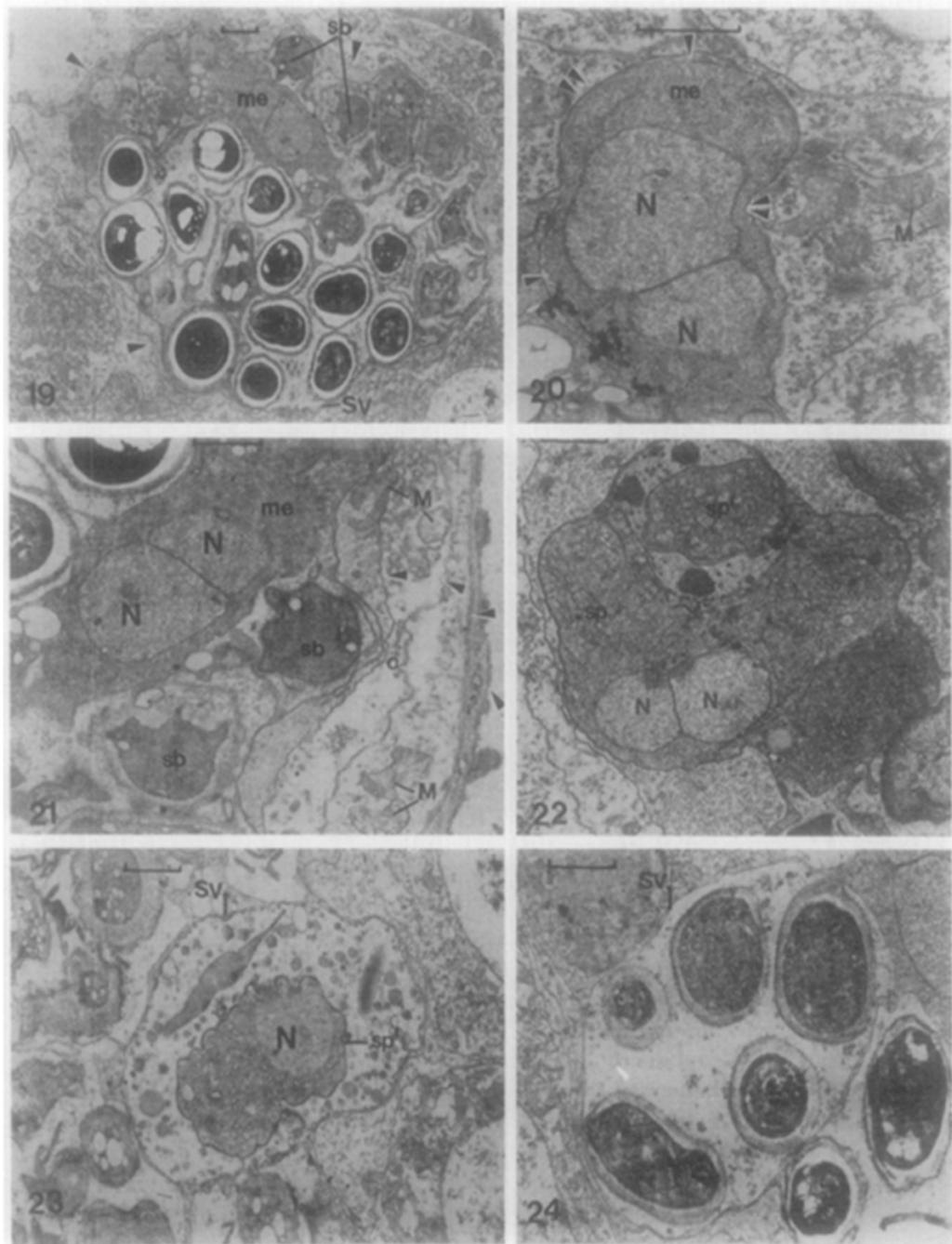
Meronts had unthickened unit membranes at the surface, the cytoplasm contained endoplasmic reticulum and abundant ribosomes, and the diplokaryotic nuclei

contained fine granular material without condensed chromatin and appeared pale against the dense cytoplasm (Figs. 19–21, me).

Early stages of sporogony were rare but may be represented by a diplokaryotic stage (Fig. 22, sp') showing an increase in endoplasmic reticulum, signs of nuclear activity in the form of condensed chromatin close to the nuclear envelopes, and some thickening of the surface membrane. The mode of formation of the sporophorous vesicle was not observed. Sections of lobed sporonts within sporophorous vesicles (Figs. 22, 23 sp') showed the thickened surface membrane, the single nucleus, and the metabolic products in the sporophorous vesicle. The metabolic products took the form of large electron-dense homogeneous globules, small globules with electron-dense borders, and fine granules (Fig. 22) all of which gradually diminished until their virtual disappearance at the time of spore



Figs. 3-18. Stages of *Amblyospora* sp. in Giemsa stained smears. Scale bar on Fig. 18 applied to all figures. Fig. 3. Young diplokaryotic meront. Fig. 4. Meront with dividing nuclei. Fig. 5. Meront with two diplokarya. Fig. 6. Meront showing fusion at the two nuclei of the diplokaryon. Fig. 7. Meront with two enlarged diplokarya: division products of these stages will be sporonts. Figs. 8,9. Early sporonts showing fusion of nuclei and mingling of chromosomes. SV, sporophorous vesicle. Figs. 10,11. Binucleate sporonts. Figs. 12,13. Quadrinucleate sporonts. Fig. 14. Octonucleate sporont. Figs. 15,16. Eight uninucleate sporoblasts within sporophorous vesicle. Figs. 17,18. Groups of eight spores.



FIGS. 19-24. Electron micrographs of *Amblyospora* sp. Scale bars = 1 μm .

FIG. 19. A meront (me) together with sporoblasts (sb) and spores in sporophorous vesicles, all surrounded by a membrane (arrowheads), possibly representing the unit membrane of a single host cell.

FIG. 20. Diplokaryotic meront. The unthickened plasma membrane is visible in places (single arrowheads) and elsewhere abuts on to the membranes (double arrowheads) of host cells containing mitochondria (M). N, parasite nuclei.

FIG. 21. Enlargement of part of Fig. 19 showing meront with diplokaryotic nuclei (N) and sporoblasts (sb) surrounded by a series of membranes (arrowheads) of host origin. Degenerate structures (M) may be host cell mitochondria.

maturity. Sporoblasts were often crenated (Fig. 21).

Immature spores (Fig. 24) showed the exospore composed of two electron-dense membranous layers at the surface and an amorphous layer of moderate electron density bounding the cytoplasmic structures. Some electron-dense granules at one pole may represent the Golgi secretions prior to the formation of the posterior vacuole. During maturation the electron-lucent endospore was interpolated between the exospore and the cytoplasm (Fig. 25), after which the amorphous layer of the exospore became less dense and was often difficult to resolve (Figs. 26,27). A layer of fine electron-dense granules deposited on the exospore, forming a fuzzy coat, may have been derived from the remains of the metabolic products. The polaroplast was well-developed, with two distinct regions (Fig. 26): an anterior part of close-packed lamellae and a posterior part of expanded sacs. The sacs were interspersed with lamellae in the transition area. The polar filament was anisofilar making five or six wide coils and four of five narrow coils (Fig. 27). The single nucleus, surrounded by concentric layers of endoplasmic reticulum, lay toward the posterior end of the spore just anterior to a membrane-bound electron-dense organelle, possibly representing the posterior vacuole (Fig. 27).

DISCUSSION

The microsporidium infecting the nerve ganglia of *C. pipiens* exhibited the developmental and morphological characters of the

genus *Amblyospora*. Sporogony was octosporous, the spores were broadly oval when fresh but truncate at both ends when stained, the exospore with amorphous and membranous components overlay the electron-lucent endospore, the polar filament was anisofilar, and the polaroplast was composed of lamellar and sac-like regions. Transovarial transmission observed in many *Amblyospora* spp. can also be inferred for the present species, as egg rafts collected from the infection site and allowed to hatch in the laboratory gave rise to infected larvae, although the possibility of transovum transmission cannot be discounted. It is concluded that the species in *C. pipiens* belongs to the genus *Amblyospora*.

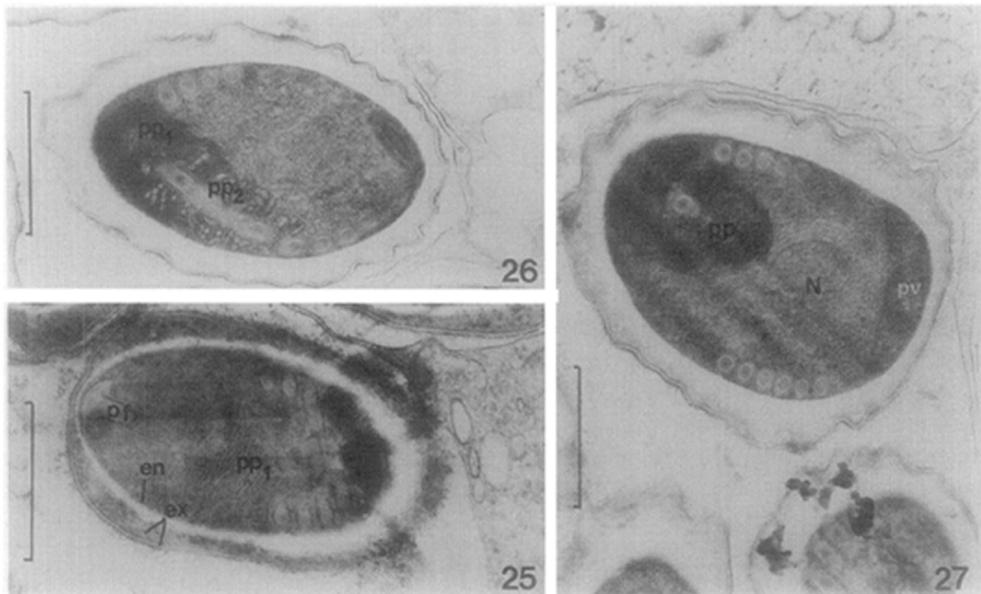
The mode of transmission of this species within generations has not been investigated. The development may be similar to that described recently for other species of *Amblyospora* (Sweeney et al., 1985, 1988); Andreadis, 1988, 1989; Becnel, 1989; Dickson, 1988), in which the mosquito larvae become infected by ingesting spores from a copepod alternate host. Stages in these horizontally infected larvae are limited to diplokaryotic meronts, usually in adipose tissue and oenocytes: sporogony, culminating in binucleate spores, is delayed until the adult stage. The binucleate spores, which hatch in situ to infect the ova, are responsible for transovarial transmission to the filial larvae, in which massive infections in the fat body ensue to give rise to uninucleate octospores, which are typically fatal to the larvae and infective to copepods.

If the same cycle occurs in the species

FIG. 22. Early sporont (sp) with thickened membrane and condensed chromatin at the edge of the nuclei (N); a sporont (sp'), in a sporophorous vesicle, containing metabolic products in the form of homogeneous electron dense globules, small granules with dense borders, and very fine granules, appears degenerate.

FIG. 23. Part of a sporont (sp'), probably a uninucleate (N) lobe of a dividing sporont, showing thickening of the surface membrane. The metabolic products in the sporophorous vesicle (SV) are less closely packed than in Fig. 22.

FIG. 24. Immature spores showing sections of the polar filament and electron dense granules which may be part of the posterior vacuole. The sporophorous vesicle (SV) now contains only a few remains of the metabolic products.



FIGS. 25–27. Electron micrographs of spores of *Amblyospora* sp. Scale bars = 1 μm .

FIG. 25. Oblique longitudinal section showing exospore (ex) made up of two membranous layers overlain by a fuzzy surface coat and subtended by an amorphous layer, electron lucent endospore (en), and polar filament (pf) passing through tightly packed lamellae of the anterior region of the polaroplast (pp₁).

FIG. 26. Oblique longitudinal section showing layers of the spore wall as in Fig. 25 (amorphous layer of exospore barely visible) and polar filament passing through lamellar (pp₁) and vesicular (pp₂) layers of polaroplast.

FIG. 27. Longitudinal section showing layers of the spore wall as in Fig. 25, 5.5 broad coils and 4.5 narrow coils of the polar filament, lamellar polaroplast (pp₁), single nucleus (N), surrounded by endoplasmic reticulum, and dense posterior vacuole (pv).

found in *C. pipiens*, only the meronts and octosporous stages in the F₁ generation have been seen. Extensive examination of tissues of overtly infected larvae and adults of *C. pipiens* revealed infections only in nervous tissue. If this species follows the pattern described above, then in the previous generations of larvae, sporogony at least must have occurred outside the nervous tissue in order to ensure that the ova became infected. It thus seems unlikely that this is the case for this *Amblyospora* species.

The present *Amblyospora* sp. is unusual in two respects: only nervous tissue, not fat body, is infected, and the larvae are not killed but survive to adulthood. *Vavraia holocentropi* invades nervous tissue as one of its sites of development (Larsson, 1986) but

in contrast to the present species is found not only in the neuropile but also in the perineurium and the nerve cell bodies. Microsporidia entirely restricted to nervous tissue have rarely been found in insects. One of these was reported by Carter (1976) in *T. paludosa* larvae collected from a hill pasture in northeast England. No details were given from which a generic diagnosis can be made. Also in *T. paludosa*, the nerve ganglia were enlarged and milky-white in appearance, but these are common signs of microsporidial infection due to massive spore accumulations in the tissue and cannot be used to differentiate species. However, the spores of the species in *T. paludosa* measured $3.5 \pm 0.06 \times 2.86 \pm 0.04 \mu\text{m}$ and, as such, are barely more than half the size of those of the present species.

Hazard and Lofgren (1971) used a *Nosema* sp. derived from a laboratory colony of *Anopheles stephensi*, in which it produced disseminated infections to test infectivity to four other mosquitoes, *Anopheles quadrimaculatus*, *C. quinquefasciatus*, *Culex salinarius*, and *Aedes aegypti*. In *A. aegypti* the infection was restricted to the nerve cell bodies in brain and nerve ganglia. The authors used this to warn against differentiating microsporidian species on the basis of tissue specificity. Whether *C. pipiens* is the optimal host of the present species is not known. Many of the developmental stages when examined at the ultrastructural level appeared vaculated side by side with well-fixed normal stages which then seemed to degenerate, although mature and apparently normal spores were produced. This suggests that *C. pipiens* may be an accidental host for a microsporidium that had infected other hosts in the collection site.

Stained slides are in the collection of the first author.

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