J. Protozool., 33(4), 1986, pp. 462–467

Pyrotheca hydropsycheae N. Sp., a Microsporidian Parasite of Caddis Fly Larvae, *Hydropsyche siltalai* Döhler, 1963 (Trichoptera, Hydropsychidae)[1]

W.-D. XIE[2] and ELIZABETH U. CANNING

Department of Pure and Applied Biology, Imperial College, London SW7 2AZ, U.K.

ABSTRACT. *Pyrotheca hydropsycheae* n. sp. is described from caddis fly larvae, *Hydropsyche siltalai* Döhler, 1963. All stages were found in oenocytes and fat body cells. Meronts were uni- or binucleate with simple surface membranes. The sporogonic stages were recognized ultrastructurally by the separation of an envelope, the sporophorous vesicle, from their surfaces. Mature sporogonial plasmodia were tetranucleate and gave rise by longitudinal fission to four uninucleate elongate sporoblasts with polar nuclei. Spores were lageniform with an inflated posterior end, containing the polar tube coils and the nucleus, and a narrow anterior section comprising two-thirds of the length, containing the polaroplast and straight part of the polar tube. The polaroplast consisted of an anterior region of loosely packed membranes arranged as partitions at angles to one another and a posterior region of increasingly closely packed parallel membranes. The spore wall consisted of an electron-dense exospore with a fuzzy coat and a thin electron-lucent endospore. All four spores derived from a sporont faced in the same direction in the sporophorous vesicle. Spores measured 8.7 μm long and extruded polar filaments were about 20 μm.

PYROTHECA incurvata was described from the copepod *Megacyclops viridis* (2) as the type species of a new genus *Pyrotheca* Hesse, 1935. Hesse suggested that four previously described microsporidia, namely *Microsporidium acuta* Moniez, 1887, *Nosema virgula* Moniez, 1887, *Gurleya francottei* Léger & Duboscq, 1909, and *Stempellia magna* Kudo, 1925, belonged to the same genus. The generic diagnosis was based solely on spore shape—elongate in the form of a powder flask (poire à poudre), one end inflated, the other more or less pointed. Although not included in the generic diagnosis, the tetrasporoblastic sporogony was clearly described. Jirovec (3) did not accept that *P. incurvata* was distinct from *Gurleya cyclopis* Leblanc, 1930, which had been referred to the other known tetrasporoblastic genus, but Poisson (8) regarded the shape of the spores as an important character in the differential diagnosis of these genera and upheld *Pyrotheca* as a valid genus. At the same time, he apparently accepted that *Pyrotheca incurvata* was identical with *Gurleya cyclopis*, and this was accepted by Sprague (9), who gave *Pyrotheca cyclopis* as the type species and *P. incurvata* as a synonym. Since then, *Pyrotheca cuneiformis* with a variable number of sporoblasts (15–21) within a pansporoblast membrane and a tetrasporoblastic *Pyrotheca* sp. have been rather incompletely described by Maurand, Fize, Michel & Fenwick (6). Recently Ovcharenko & Palienko (7) reported the occurrence of *P. cuneiformis* and *P. virgula*, as well as *Microsporidium cyclopis* (Kudo, 1921) and an unnamed species of *Stempellia* in several copepods in the USSR.

During autumn 1983 and spring 1984, a population of caddis fly larvae, *Hydropsyche siltalai* Döhler, 1963, was found to be infected with a tetrasporoblastic microsporidium with lageniform spores. The prevalence was about 6%. The species has been studied by light and electron microscopy and is ascribed to the genus *Pyrotheca*. The material has enabled us to characterize the genus at the ultrastructural level.

MATERIALS AND METHODS

The larvae of *H. siltalai* were collected from the river Wey at Tilford, Hampshire, U.K.

For light microscopy, smears made from the fat tissue of infected larvae were dried in air, fixed in methanol, and stained in 10% Giemsa stain at pH 6.8.

For electron microscopy, pieces of fat body were dissected into 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 10 min at room temperature and then fixed an additional hour at 4°C. After washing in buffer, the tissue was post-fixed in 1% (w/v) OsO_4 in buffer for 1 h at 4°C. Tissues were then block-stained in 0.25% (w/v) aqueous uranyl acetate, dehydrated in acetone, and embedded in Spurr's resin. The sections were stained in 1% uranyl acetate and in Reynold's lead citrate and were examined in a Philips 300 electron microscope at an accelerating voltage of 80 kV.

RESULTS

Light microscope observations. Oenocytes and fat body cells are the primary sites of infection. Meronts are uninucleate, rounded stages which become binucleate in preparation for division by binary fission (Fig. 1). They measure 3.4 ± 0.6 μm (n = 35) and 4.4 ± 0.4 μm (n = 35) respectively.

Some binucleate stages, instead of dividing, enlarge further (Fig. 2) and undergo a second nuclear division giving rise to tetranucleate sporogonial plasmodia. The four nuclei of the plasmodia first take up peripheral positions in a Maltese cross arrangement (Figs. 1, 3), then rearrange themselves along one edge (Figs. 4, 5).

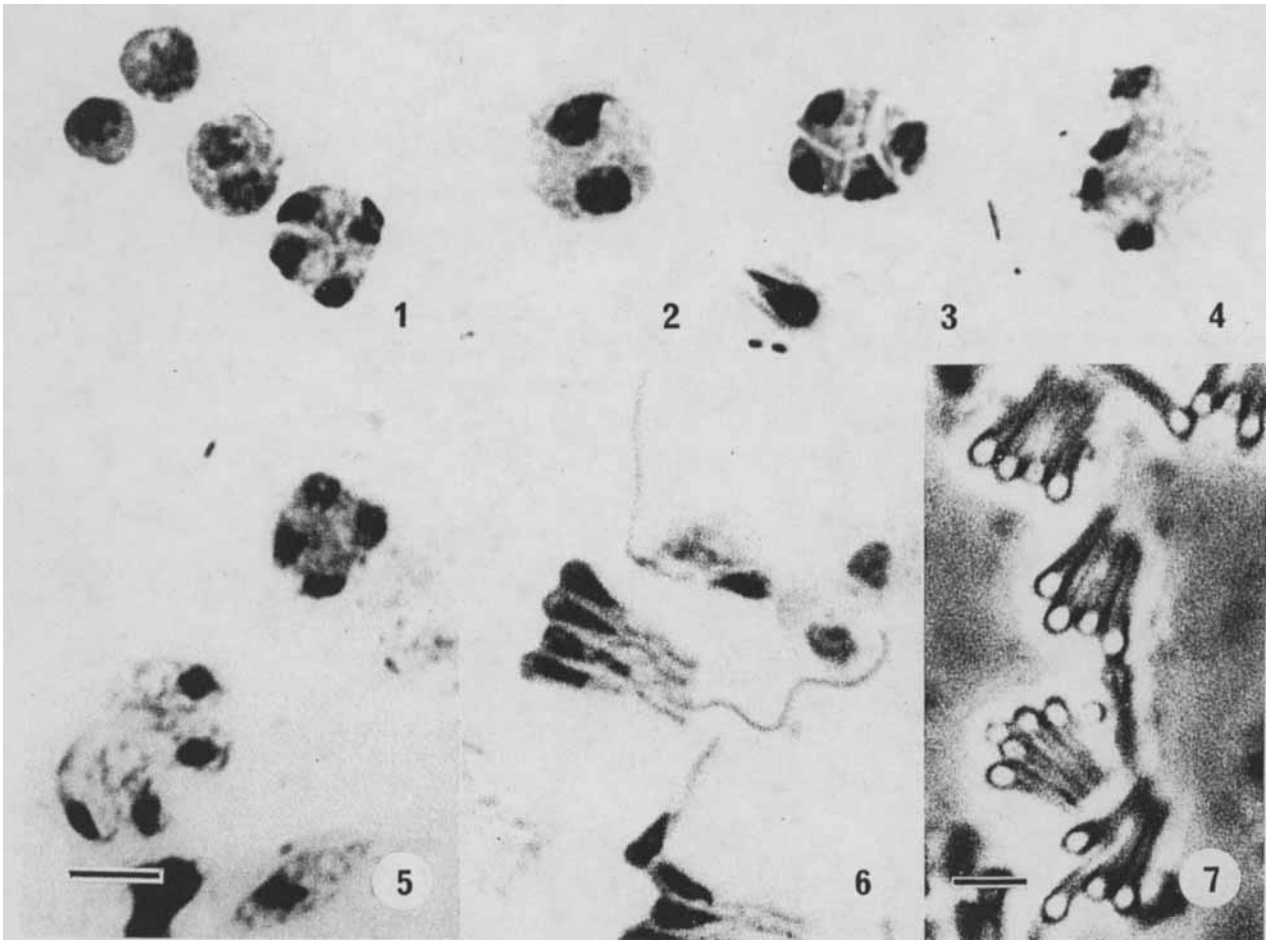
The mature spores lie packed together side by side, all facing in the same direction (Fig. 6). One end of the spore is somewhat inflated, stains deeply, and contains the nucleus. The spore narrows towards the faintly-staining anterior end which contains the polaroplast. Polar tubes were frequently found extruded from the narrow end. Stained spores measured 8.7 ± 0.5 μm long; the inflated region measured 2.0 ± 0.3 μm in diameter, and the neck region 1.3 ± 0.1 μm (n = 35). The corresponding measurements of fresh spores (Fig. 7) were 9.32 ± 0.3 μm, 2.2 ± 0.1 μm, and 1.4 ± 0.1 μm.

Electron microscope observations. The meronts lie in direct contact with the host cell cytoplasm and are surrounded by a simple unit membrane. The isolated nuclei frequently contain electron-dense material, probably condensed chromatin, and possibly also a nucleolus. Cisternae of endoplasmic reticulum, predominantly smooth, are arranged irregularly in the cytoplasm (Figs. 8, 9).

The first sign of transition to the sporogonic cycle is the appearance around the cells of an additional envelope in the form of small blisters pushing away from the unit membrane (Fig. 10). These are first seen around uninucleate sporonts distinguishing them from uninucleate meronts. The separation of the outer envelope continues, ultimately to form a complete sporophorous vesicle, while the sporont undergoes nuclear division to produce a tetranucleate sporogonial plasmodium. Synapto-

[1] This work was carried out during tenure of a Zhongshan University Postgraduate Fellowship by W.-D. Xie. We are grateful to Dr. M. I. Crichton of the Department of Zoology, University of Reading, England, for identifying the caddis fly larvae.

[2] Present address: Institute of Entomology, Zhongshan University, Guangzhou, People's Republic of China.



Figs. 1–7. Light micrographs of *Pyrotheca hydropsycheae*, Giemsa-stained except Fig. 7, which is a fresh preparation. Scale bars = 5 μm. The bar on Fig. 5 represents the scale for Figs. 1–6. **1.** Uninucleate and binucleate meronts and a tetranucleate sporont. **2.** Enlarged binucleate stage, probably a sporont. **3.** Division of tetranucleate sporont into sporoblasts; the nuclei have taken up peripheral positions. **4, 5.** Tetranucleate sporonts; typically the nuclei assume positions along one edge. **6.** Groups of four spores, some of which have extruded their polar tubes. **7.** Fresh spores in groups of four.

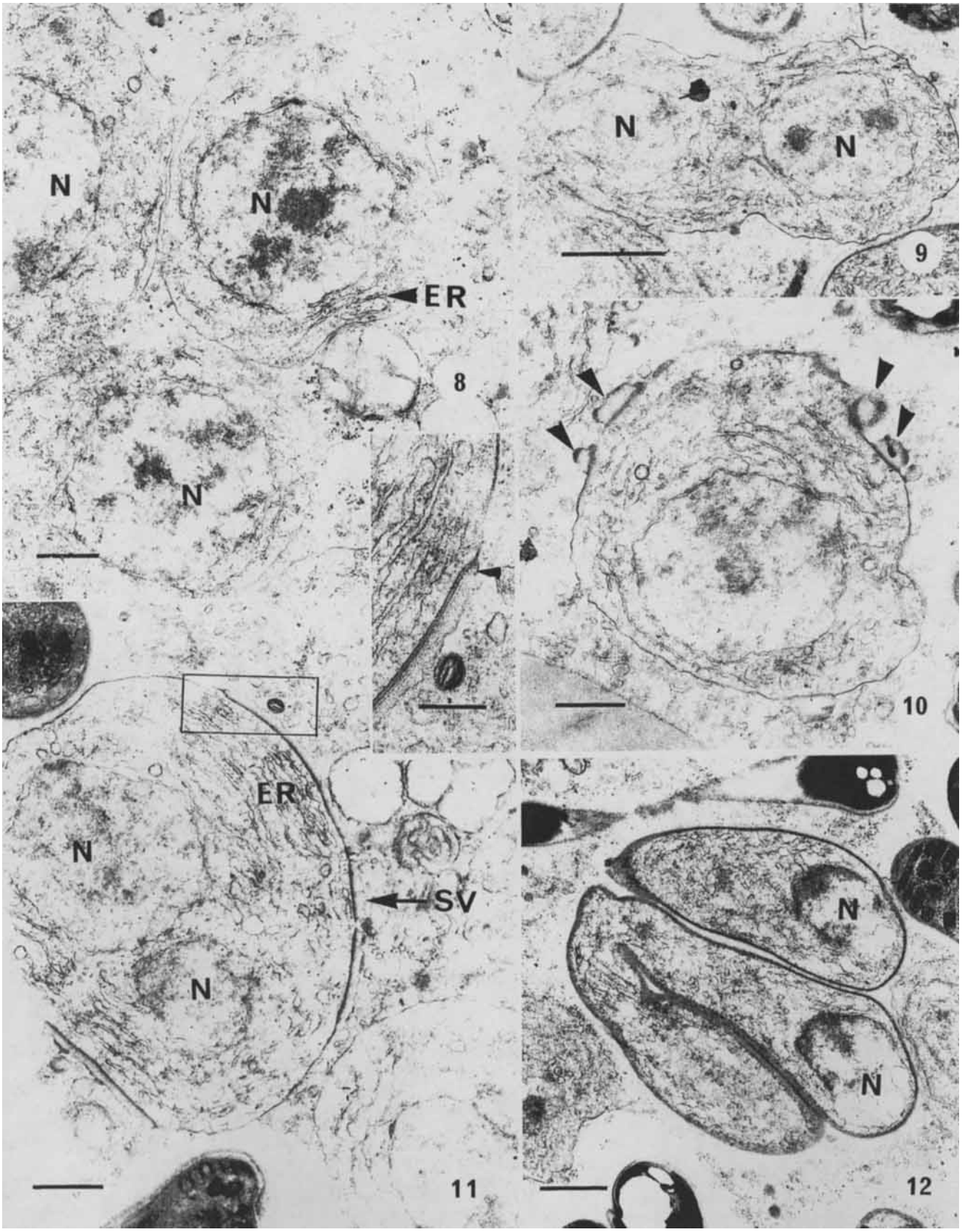
nemal complexes indicative of meiosis were not seen. Within the vesicle, the plasmodial surface is thickened by the addition of at least two layers external to the unit membrane (Fig. 11, insert). The amount of endoplasmic reticulum increases and includes numerous expanded vesicles. The sporophorous vesicle remains fine but persists throughout sporogony and during the maturation of the spores. At first the vesicle contains a uniform, very finely granular secretion, but this disappears during the maturation processes.

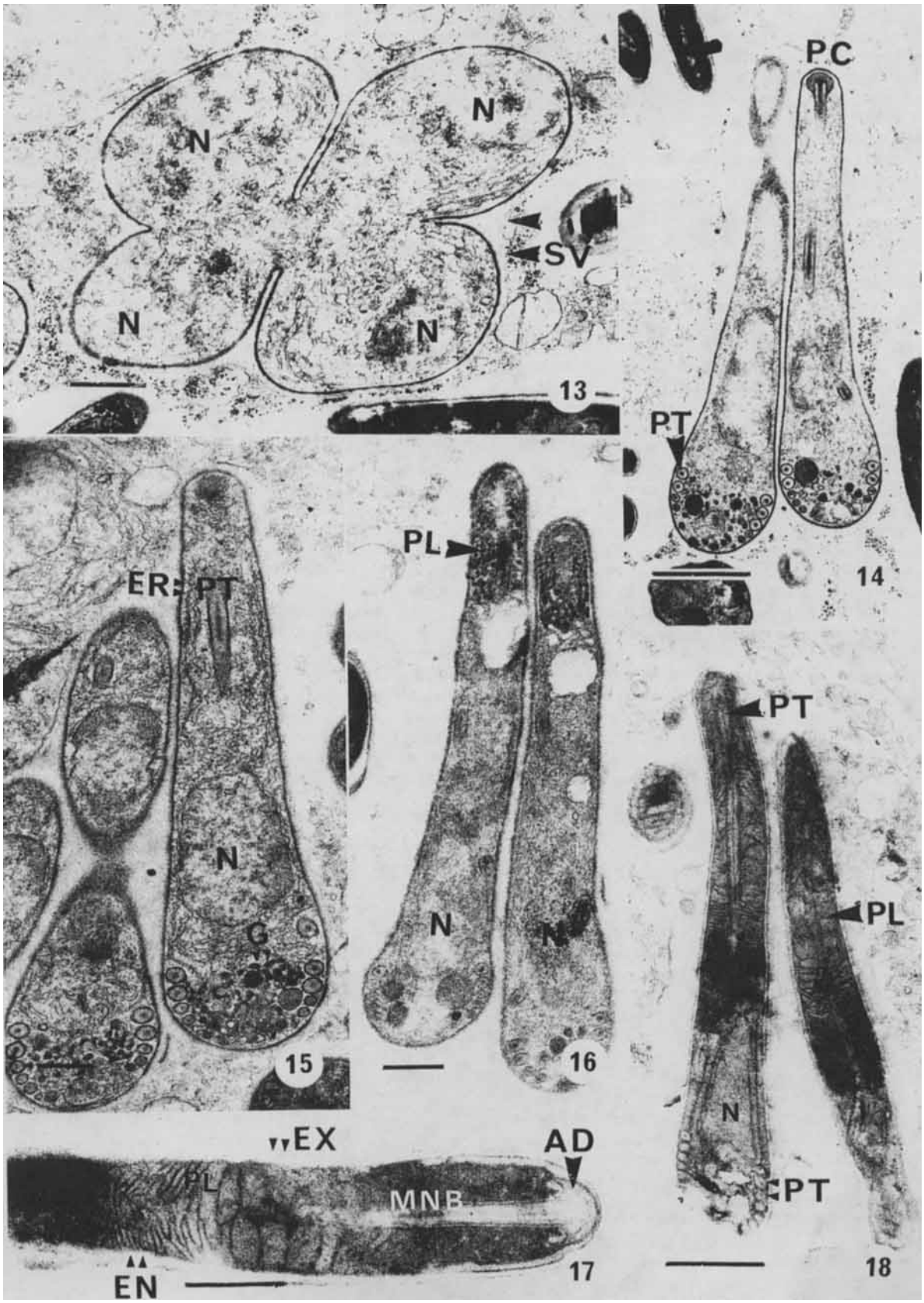
Longitudinal division of the mature sporogonial plasmodium

→

Figs. 8–12. Electron micrographs of *Pyrotheca hydropsycheae*. N, nucleus; ER, endoplasmic reticulum; SV, sporophorous vesicle. **8.** Uninucleate meronts showing simple plasma membranes. Scale bar = 0.5 μm. **9.** Binucleate meront about to divide. Scale bar = 0.5 μm. **10.** Uninucleate sporont. Several small blisters (arrows) have appeared at the surface; they have been formed by the separation of an envelope, the future sporophorous vesicle, from the plasma membrane. Scale bar = 1 μm. **11.** Binucleate sporont; the sporophorous vesicle is now more extensive (arrow), and beneath it the plasma membrane of the sporont has been thickened. The area enclosed in the rectangle is enlarged in the inset, which shows the sporophorous vesicle (arrow) and resolves the membrane thickening into at least two layers. Scale bars = 1.0 μm and 0.5 μm (inset). **12.** Longitudinal section of dividing sporont with three of the four sporoblasts visible; the nuclei have terminal positions. Scale bar = 1 μm.

Figs. 13–18. Electron micrographs of *Pyrotheca hydropsycheae*. AD, anchoring disc; EN, endospore; ER, endoplasmic reticulum; EX, exospore; G, Golgi network; MNB, manubroid region of polar tube; N, nucleus; PC, polar cap; PL, polaroplast; PT, polar tube. **13.** Transverse section of dividing sporont completely surrounded by the sporophorous vesicle. Scale bar = 1 μm. **14–16.** Sporoblasts in longitudinal section. The polar tube arising in a coil from the posteriorly located Golgi network is also visible in its straight anterior region joining to the polar cap. The nucleus is located in the expanded posterior region. Scale bars: Fig. 14 = 1 μm; Figs. 15, 16 = 0.5 μm. **17.** Anterior end of a mature spore. This region is occupied by the polaroplast with its open and closely packed membranes and the manubroid part of the polar tube joining the umbrella-shaped anchoring disc. Scale bar = 1 μm. **18.** Two of the four spores in a sporophorous vesicle. The nucleus occupies the region just anterior to the coils of the polar tube, and the spore narrows in front of the nucleus where the straight part of the polar tube runs through the polaroplast. The spore on the right shows the point where the polar tube curves diagonally across the spore from the coil to the straight part. Scale bar = 0.5 μm.


N
N
ER
8
N
N
N
9
10
ER
N
SV
N
N
N
11
12


N
N
N
N
SV
13
PC
PT
14
ER
PT
N
G
15
PL
N
N
16
PT
PL
N
PT
EX
AD
PL
MNB
EN
17
18

occurs after the nuclei have moved towards one pole. Three of the forming sporoblasts are illustrated in Fig. 12 in longitudinal section, and the four sporoblasts in transverse section through the nucleated region are seen in Fig. 13. These show that the sporoblasts are separated in a cluster rather than in one plane, as suggested by the light micrographs.

Developing sporoblasts are illustrated in Figs. 14, 15. At the posterior inflated end there is a prominent reticulum, probably representing the Golgi apparatus, which gives rise to the coils of the polar tube. Some large membrane-bounded vesicles in this region appear to be secretions from the same system. At the point where the spore begins to narrow lies the nucleus, now without condensed chromatin or nucleolus, surrounded by endoplasmic reticulum. The straight region of the polar tube runs through the center of the narrow region of the spore to join with the polar cap. The electron-dense core of the polar tube expands at the anterior tip of the tube beneath the dome-shaped polar cap. In some sporoblasts the primordia of the polaroplast are seen as electron-dense vesicles surrounding the polar tube at the anterior end (Fig. 16). The spore wall, at the sporoblast stage, consists only of the electron-dense coat derived from the layers deposited on the surface of the sporogonial plasmodium.

In more mature spores (Figs. 17, 18), the polaroplast occupies the entire narrow region around the polar tube. It consists of an anterior region, where the membranes are loosely arranged as a series of partitions at angles to one another and a posterior region where the membranes are stacked in parallel array, becoming more closely packed towards the nucleus. The membranes appear to arise from the outer wall of the polar tube. The polar cap is similar in organization to that seen in sporoblasts but extends back under the spore wall (Fig. 17). There are six or seven coils of the polar tube at the posterior end, where the wall often collapses inwards in fixed spores. The nucleus is triangular in section and occupies the region between the polar tube coils and the polaroplast. The spore wall is thin compared with that of many microsporidia, being composed of a poorly developed endospore and an exospore overlain by a fuzzy coat. The latter could have been derived from the finely granular contents of the sporophorous vesicle.

DISCUSSION

Two genera of microsporidia have been described with tetrasporoblastic sporogony, namely *Gurleya* Doflein, 1898 and *Pyrotheca* Hesse, 1935. The type species of *Gurleya, G. tetraspora* Doflein, 1898, was described from *Daphnia* spp. (Cladocera) insufficiently well for a firm distinction to be drawn between it and *Pyrotheca*; *Gurleya* was characterized by pyriform spores and *Pyrotheca* by elongate spores in the shape of a powder flask. The type species of *Gurleya* has not been examined by electron microscopy, but Loubès & Maurand (4) and Loubès, Maurand & Rousset-Galangau (5) described the ultrastructure of the sporogonic stages of *Gurleya chironomi* Loubès & Maurand, 1975 from a chironomid larva, *Orthocladius* sp. Particular features of note are the synaptonemal complexes in the nuclei of uninucleate sporonts and the formation of a thick, persistent sporophorous vesicle enclosing small and large electron-dense secretory granules. The spores are ovoid.

The present ultrastructural study of a tetrasporoblastic microsporidium from caddis fly larvae shows that the species is characterized by a very fine sporophorous vesicle enclosing a barely visible, almost homogeneous secretion, differing strikingly from the large secretory granules described in *G. chironomi*. The spore shape is also entirely different. Furthermore we found no evidence of meiosis during sporogony although its occurrence cannot be entirely ruled out. Although the ultrastructural details for *Gurleya* have not been given for the type species, we believe there is justification, at least at present, for retaining the genus *Pyrotheca*, and we have assigned the parasite of *H. siltalai* to it. The parasite, producing a variable number of sporoblasts and named *Pyrotheca cuneiformis* by Maurand, Fize, Michel & Fenwick (6), probably does not belong to this genus.

More difficult is the differentiation of the genera *Pyrotheca* and *Cougourdella* both described by Hesse from copepods in the same publication (2). The spore shape was similar but *Pyrotheca* was shown to be tetrasporoblastic while it was said of *Cougourdella* that the sporont gave rise to two or even a single sporoblast. Unfortunately the two species *C. magna* and *C. pusilla* have not been observed since, and Hesse's observations have not been confirmed. The parasite of *H. siltalai* clearly resembles the genus *Pyrotheca*. It differs in size and/or shape of spores from the previously described species and is here named as a new species, *P. hydropsycheae*.

Recently Sweeney, Hazard & Graham (10) and Andreadis (1) have described the development of *Amblyospora* spp., respectively, from the mosquitoes *Culex annulirostris* and *Aedes cantator* in copepods, which act as alternate hosts. In both cases the spores in the copepods superficially resembled those of *Pyrotheca*. Andreadis discussed the possibility that species of *Pyrotheca* may actually represent intermediate stages of *Amblyospora* and that *Pyrotheca* Hesse, 1935 would have priority over *Amblyospora* Hazard & Oldacre, 1975 if they were synonymized; however, both Sweeney et al. (10) and Andreadis (1) indicated that in the *Pyrotheca*-like parasites of copepods the sporophorous vesicle separates from the sporoblast surface after completion of the sporogonial divisions and thus that the spores develop individually within fine sporophorous vesicles, not in groups of four as Hesse described. Also the spores in copepods differ in shape and structure, particularly in the form of the polaroplast, from the spores we found in *H. siltalai*. The present ultrastructural study has shown that the parasite of *H. siltalai* is tetrasporoblastic and develops in sporophorous vesicles. It appears therefore that the *Pyrotheca*-like stages of *Amblyospora* are distinct and that the genus *Amblyospora* is valid.

Pyrotheca hydropsycheae n. sp.

Type host. Hydropsyche siltalai Döhler, 1963 (Trichoptera, Hydropsychidae).

Type locality. River Wey at Tilford, Hampshire, U.K.

Merogony. Uninucleate meronts divide by binary fission.

Sporogony. Division of a uninucleate sporont within a sporophorous vesicle gives rise to a tetranucleate sporont, then to a cluster of uninucleate sporoblasts with polar nuclei.

Spores. Uninucleate, lageniform, 8.7 μm long, all facing in the same direction in the sporophorous vesicle. Polaroplast occupying the narrow region of the spore and consisting of an anterior region of loosely packed membranes arranged as partitions at angles to one another and a posterior region of increasingly closely packed parallel membranes. Six or seven coils of the polar tube, which measures about 20 μm when extruded.

LITERATURE CITED

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

2. Hesse, E. 1935. Sur quelques microsporidies parasites de *Megacyclops viridis* Jurine. *Arch. Zool. Exp. Gén.*, **75**: 651–661.

3. Jírovec, O. 1936. Studien über Microsporidien. *Věstn. Česk. Spol. Zool.*, **4**: 1–75.

4. Loubès, C. & Maurand, J. 1975. Etude ultrastructurale de *Gurleya chironomi* n. sp. Microsporidie parasite des larves d'*Orthocladius* (Diptera—Chironomidae). *Protistologica*, **11**: 233–244.

5. Loubès, C., Maurand, J. & Rousset-Galangau, V. 1976. Présence de complexes synaptonématiques dans le cycle biologique de *Gurleya chironomi* Loubès et Maurand, 1975: un argument en faveur d'une sexualité chez les Microsporidies? *C. R. Acad. Sci. Paris*, **282**: 1025–1027.

6. Maurand, J., Fize, A., Michel, R. & Fenwick, B. 1972. Quelques données sur les microsporidies parasites de copépodes cyclopoides des eaux continentales de la région de Montpellier. *Bull. Soc. Zool. France*, **97**: 707–717.

7. Ovcharenko, N. A. & Palienko, L. P. 1982. Microsporidian infection of cyclopoid copepods from ponds of Kiev. *Journal of Hydrobiol. Kiev.*, **18**: 80–83. (in Russian)

8. Poisson, R. 1953. Ordre des Microsporidies, *in* Grasse, P.-P., ed., *Traité de Zoologie*, Masson et Cie, Paris, pp. 1042–1070.

9. Sprague, V. 1977. Annotated list of species of microsporidia, *in* Bulla, L. A., Jr. & Cheng, T. C., eds., *Comparative Pathobiology*, Plenum Press, New York, 2: 1–510.

10. 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.*, **45**: 98–102.

Received 14 IV 86; accepted 06 VI 86

J. Protozool., 33(4), 1986, pp. 467–472

Conditions for Maximum Enflagellation in *Naegleria fowleri*[1]

B. L. CABLE and D. T. JOHN[2]

Department of Microbiology/Immunology, Oral Roberts University, School of Medicine, Tulsa, Oklahoma 74137

ABSTRACT. Ameba to flagellate transformation in *Naegleria fowleri* (Lovell strain) was affected by growth temperature, phase of growth, strain of ameba, culture agitation, enflagellation temperature, enflagellation diluent, and cell concentration. Amebae transformed best when they were grown without agitation and enflagellated with agitation. Regardless of growth temperature (23°, 30°, 37°, and 42°C were tested), amebae transformed best at 37°C. Enflagellation was greatest for cells harvested between 24 h (mid-exponential) and 84 h (late stationary) of growth.

AMEBAE of *Naegleria fowleri*, the cause of primary amebic meningoencephalitis in man, are able to transform into transient, nonfeeding, nondividing flagellates. This transformation, or enflagellation, can be induced by removing the growth medium from cultures and replacing it with non-nutrient buffer or distilled water (6, 15).

We describe here the conditions for maximum enflagellation in *N. fowleri*. Percent enflagellation varied seven-fold among the 13 human isolates of *N. fowleri* examined. Four isolates did not enflagellate under the conditions used. Cells enflagellated best when amebae were grown without agitation and transformed with agitation. A 37°C enflagellation temperature yielded the greatest percent of flagellates.

MATERIALS AND METHODS

Ameba strains. The principal strain of *N. fowleri* used in this study was the Lovell strain, isolated in 1974 from a patient who died of primary amebic meningoencephalitis in Florida (1). The strain has been maintained in axenic culture in our laboratory since 1976.

Twelve other strains of *N. fowleri* were also used to compare percent enflagellation. Their sources and dates of isolation have been given elsewhere (6).

Cultivation. Amebae were grown axenically in Nelson medium (13) supplemented with 2% (v/v) calf serum. Tissue-culture flasks (25 cm^2, Corning Glass Works, Corning, NY) containing 10 ml of medium were inoculated with 2.5×10^5 amebae each to give an initial concentration of 10^4 amebae/cm^2 of surface area. Cell counts were made using a Coulter counter (model Z_{BI}, Coulter Electronics, Inc., Hialeah, FL) using settings previously reported (14).

Enflagellation procedure. Amebae were grown axenically under specified culture conditions of temperature and agitation to the desired phase of growth. The culture medium was poured off and the adherent amebae were washed twice with Page ameba saline (10) which had been kept at the growth temperature. Amebae were suspended in 8 ml of cold (4°C) ameba saline. A cell count was made and the suspension adjusted to the desired cell concentration. The final cell suspension was transferred to a 125-ml Erlenmeyer flask and agitated at 100 rpm in a gyratory shaker (New Brunswick Scientific, Edison, NJ) at the appropriate transformation temperature.

Time zero was defined as the point when amebae were suspended in cold ameba saline. Hourly, to 8 h, samples of the ameba suspension were fixed and stained with D'Antoni's iodine and examined by light and phase-contrast microscopy. At least 100 cells per sample were counted to determine percent flagellates. Cells were considered to be flagellates if flagella were evident, in any length or number, regardless of body shape.

Cell concentration. Amebae were grown at 37°C in agitated cultures and harvested by centrifugation (1000 *g*, 5 min, 23°C) at early stationary growth phase. Cell concentrations examined were 10^5, 10^6, 10^7, and 10^8 cells/ml of enflagellation buffer. Transformation temperature was 37°C.

Phase of growth. Amebae were cultured unagitated at 37°C and tested for enflagellation at 12, 24, 36, 48, 60, 72, 84, and 96 h of culture age. At a 37°C incubation temperature and starting with a cell density of 10^4 amebae/cm^2, the following are incubation periods with corresponding growth phases: 12 h, early exponential; 24 h, mid-exponential; 36 h, late exponential; 48 h, transition; 60 h, early stationary; 72 h, mid-stationary; 84 h, late stationary; 96 h, decline. Enflagellation temperature was 37°C and cell concentration in all samples was 10^6 cells/ml of suspension buffer.

Enflagellation buffers. The following suspension buffers were compared for their ability to promote maximum enflagellation: Page ameba saline, Tris buffer [2 mM Tris (hydroxymethyl) aminomethane-HCl], phosphate-buffered saline (PBS), physiologi-

[1] Our thanks to Pamela Ickes for technical assistance. This research was supported by Public Health Service grant AI-18788 from the National Institute of Allergy and Infectious Diseases.

[2] Corresponding author.