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Contribution to the Study of *Enterocystis racovitzai*, a Gregarine Parasite of *Baetis rhodani* (Ephemeroptera: Baetidae)

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Summary. Some ultrastructural aspects of *Enterocystis racovitzai,* a gregarine living in the midgut of the nymph of the mayfly *Baetis rhodani,* were examined. The initial development of the parasite took place in the epithelial cells and the early pairing of the individuals formed syzygies into the gut lumen. Intracellular and extracellular phases coexisted in the same host. The finding of gamonts in advanced syzygy in a cyst allowed us to highlight some organizational details of this developmental phase. Gametes in formation were also observed. The fine structure of the parasite was discussed in reference to the description reported for other gregarines.

Key words: *Enterocystis racovitzai*, gregarine, mayfly, ultrastructure.

INTRODUCTION

Several species of gregarines belonging to the genus *Enterocystis* have been described in the alimentary canal of the aquatic developmental phases of some Ephemeroptera (Schneider 1882; Zwetkow 1926; Codreanu 1940; Grassé 1953; Bobyleva, 1963 in Arvy and Peters 1973; Desportes 1963, 1964, 1966, 1974; Geus 1969; Arvy and Peters 1973; Codreanu and Codreanu-Balcescu 1979; Peters and Arvy 1979). These gregarins have been referred to Enterocystidae and Gregarinidae. According to Desportes (1966) the family Enterocystidae encompasses only the genus *Enterocystis*, the species of which parasite exclusively the gut of mayfly larvae. The representatives of this genus are characterized by the lack of a septum between proto- and deutomerite. The genera *Gregarina* and *Gamocystis* are included into Gregarinidae even though the lack of a real septum subdividing the gregarine cell of *Gamocystis ephemerae* has been considered by Rühl (1976) a valid trait to reconsider the taxonomic position of this species. The doubtful allocation of *G. ephemerae* into Gregarinidae has been previously pointed out by Codreanu (1940). Desportes (1963), on the basis of some similarities with other gregarines belonging to *Enterocystis*, proposed to attribute this species to this last genus.

As far as the occurrence of gregarines in the mayfly *Baetis rhodani* (Ephemeroptera: Baetidae) is concerned, representatives of three species of gregarines, namely *Enterocystis racovitzai*, *E. fungoides* and *E. ensis*, have

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been collected in different localities (Desportes 1963, 1964). Some ultrastructural investigations were carried out by Desportes (1974) on *E. fungoides* living in larves of no better identified Baetidae collected in the Var River (France).

In our paper we report some ultrastructural details of a gregarine the morphology of which corresponds to that of *E. racovitzai* Codreanu, 1940. The gregarine is present in various developmental phases taking place in the epithelial cells and in the lumen of the midgut of nymphs of the mayfly *B. rhodani*.

MATERIALS AND METHODS

Nymphs of *Baetis rhodani* (Pictet 1843-45), family Baetidae (Ephemeroptera) were collected in the Lemme Stream (Voltaggio, Piedmont-18/10/1996; 10/4/1998). Among 48 examined insects, gregarines belonging to *Enterocystis racovitzai* Codreanu, 1940 were found, both as intracellular young gregarine and syzygy, in the alimentary canal of 10 individuals. One of them with dark wing-pads showed a mature gametocyst in the terminal tract of the midgut.

After dissection, free gregarines were observed *in vivo* under both light and interpherence contrast microscopes.

For ultrastructural investigation, selected material was fixed according to different techniques, as follows: (a) 1 h in Karnovsky's medium (1965); (b) 1 h in glutaraldehyde diluted at 2% in Na-cacodylate buffer (0.2 M). After fixation, specimens were repeatedly rinsed in the same buffer, postfixed in 1% osmium tetroxide for 1 h at 4°C and then dehydrated in a graded ethanol series.

For observations under Scanning Electron Microscope (SEM), the samples were critical point dried using a CO_2 Pabisch CPD 750 apparatus, mounted on stubs with silver-conducting paint, coated with gold-palladium in a Balzers Union evaporator and observed under a Philips EM 505 at an accelerating voltage of 18 kV.

For observations under Transmission Electron Microscope (TEM), the material was embedded through propylene oxide in Epon-Araldite. Thin sections obtained by a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, were examined under Philips EM 400 T.

RESULTS

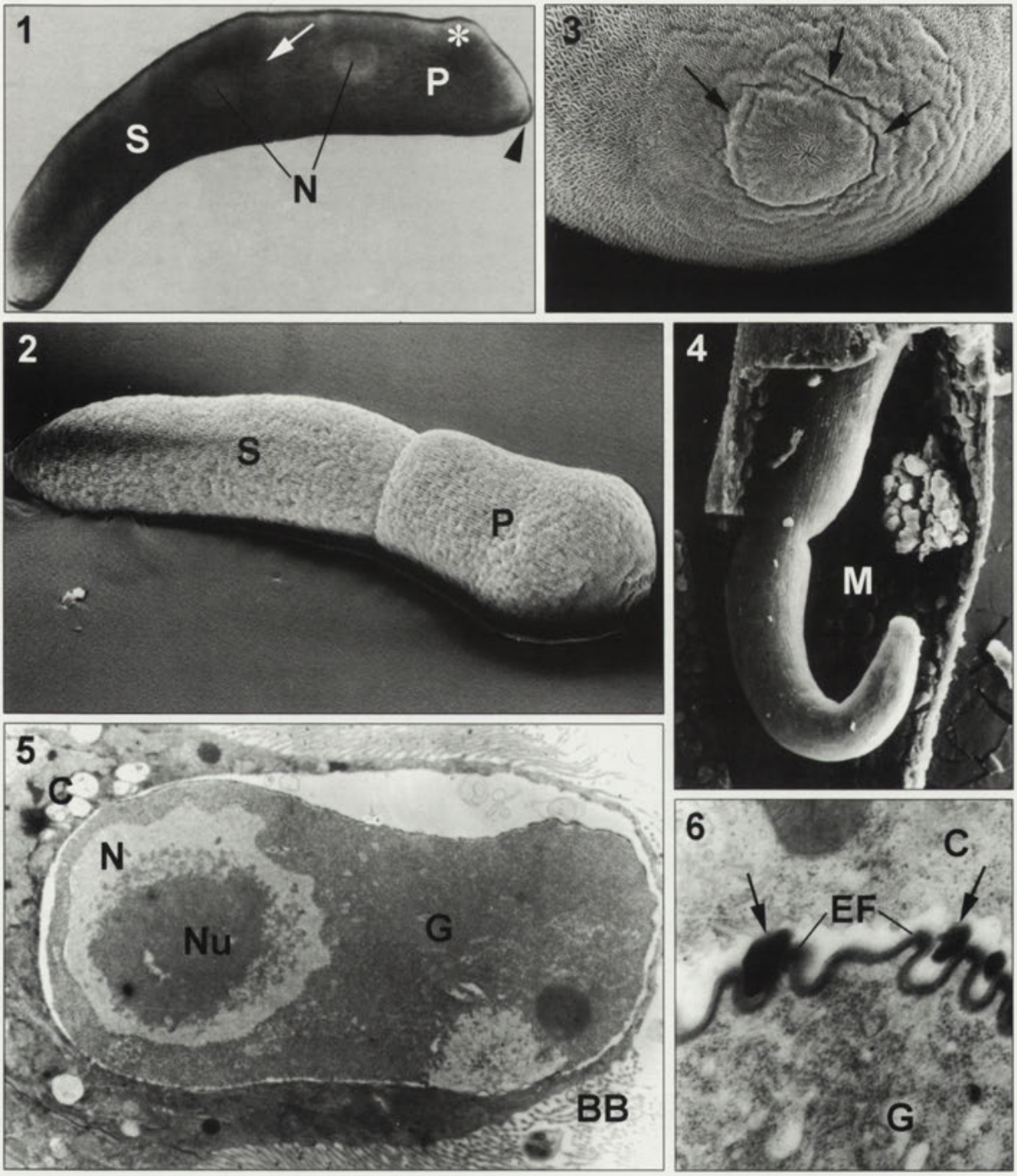
The midgut of some nymphs of *Baetis rhodani* is the site of development of gregarines belonging to *Enterocystis racovitzai*. This parasite, which shows the early pairing of the individuals to form syzygies, causes heavy infection due to the remarkable amount of gregarines into the mayfly midgut lumen. Each syzygy consists of two specimens (gamonts) in pair defined primite and satellite on the basis of their morphology (Figs. 1, 2), which varies according to the phase of differentiation. In the maturing syzygies, the satellite tends to become longer then the primite. This latter is characterized by lateral lobes and an apical protuberance (Fig. 1), which is separated from the rest of the surface by some grooves (Fig. 3).

The early development of the trophozoite takes place inside the epithelial cells of the gut and these intracellular phases of differentiation coexist with the syzygies living in the gut lumen (Fig. 4). Clearly identifiable intracellular trophozoites are individually included in vacuoles, which deform the apical part of the epithelial cell abutting inside the gut lumen (Fig. 5). At this stage of differentiation the parasite is characterized by a slightly elongated shape and a fairly homogeneous cytoplasm lacking inclusions. It measures about 14 μm in length and shows a "lobated" nucleus occupying the innermost region of the parasite. The peripheral border of the parasite forms short epicyte folds at irregular intervals and electron-dense material accumulates between contiguous epicytes (Fig. 6).

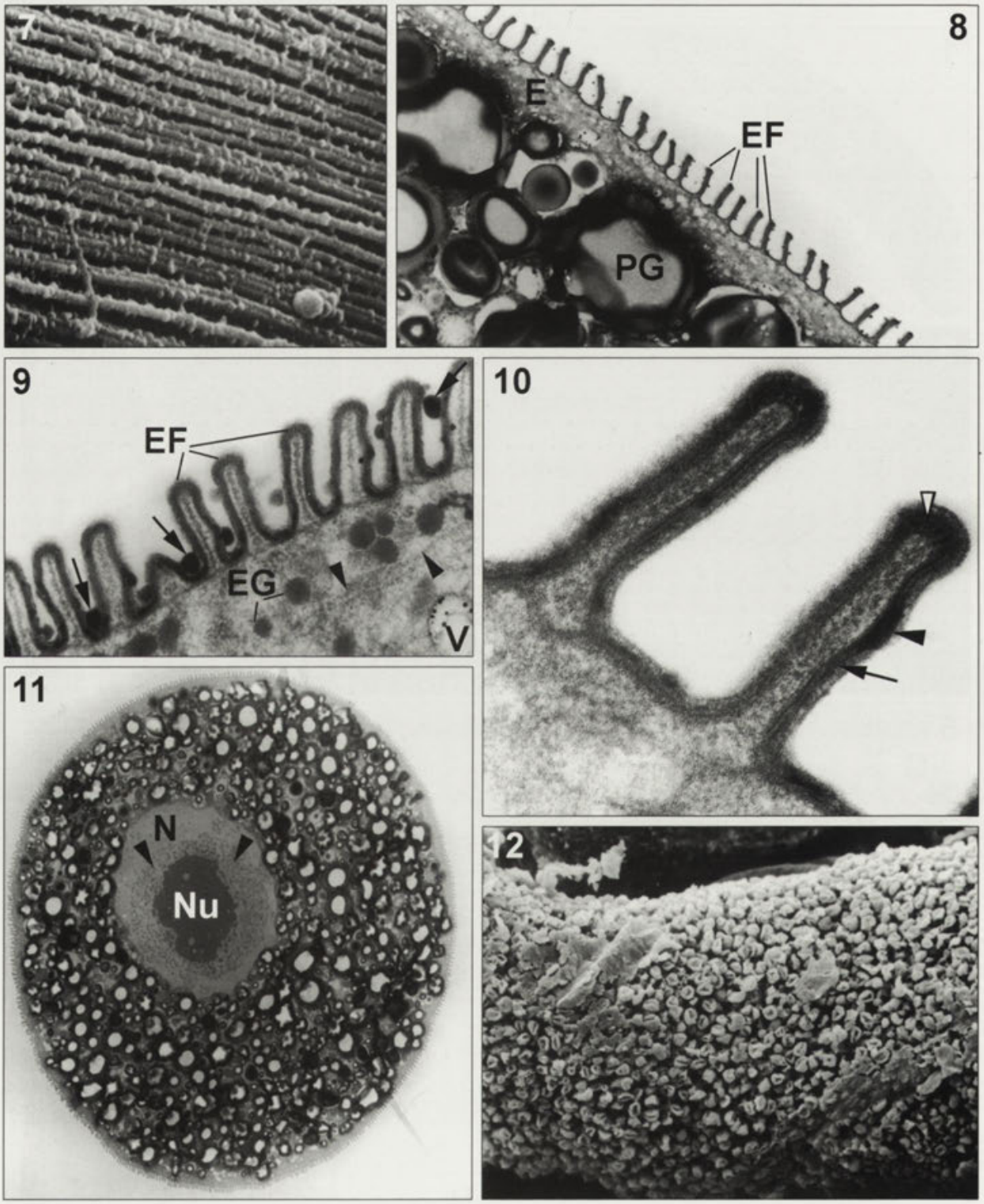
In the syzygy the entire surface of primite and satellite is delimited by an epicyte layer which forms a series of regular longitudinal folds (Fig. 7). Epicytes emerge from the thin peripheral ectocyte bounding the entocyte with its remarkable amount of paraglycogen granules (Fig. 8). Ectocyte includes vacuoles, electron-dense inclusions and fibrillar components (Fig. 9). Extracellularly, electron-dense granules gather between epicytes (Fig. 9). The epicytes (about 0.5 μm high) are bound by an electron-dense multilayered border which shows a striated appearance in the apical part of the epicyte folds (Fig. 10). On occasion, electron-dense granules adhere to the inner cytomembranes (Fig. 10). The body of each individual is completely fillep up of paraglycogen granules (Fig. 11), which are slightly interspaced in the cytoplasm (Fig. 12). The nucleus shows an irregular border and contains a nucleolus constituted by an evident central electron-dense portion and scattered filaments (Fig. 11).

The association of the gamonts to form a couple occurs along the facing epicyte folds of the posterior part of the primite and the anterior one of the satellite (Fig. 13). The epicyte folds involved in this association undergo modifications and give rise to a contact zone where the two confronting membranes maintain a parallel alignment (Fig. 14).

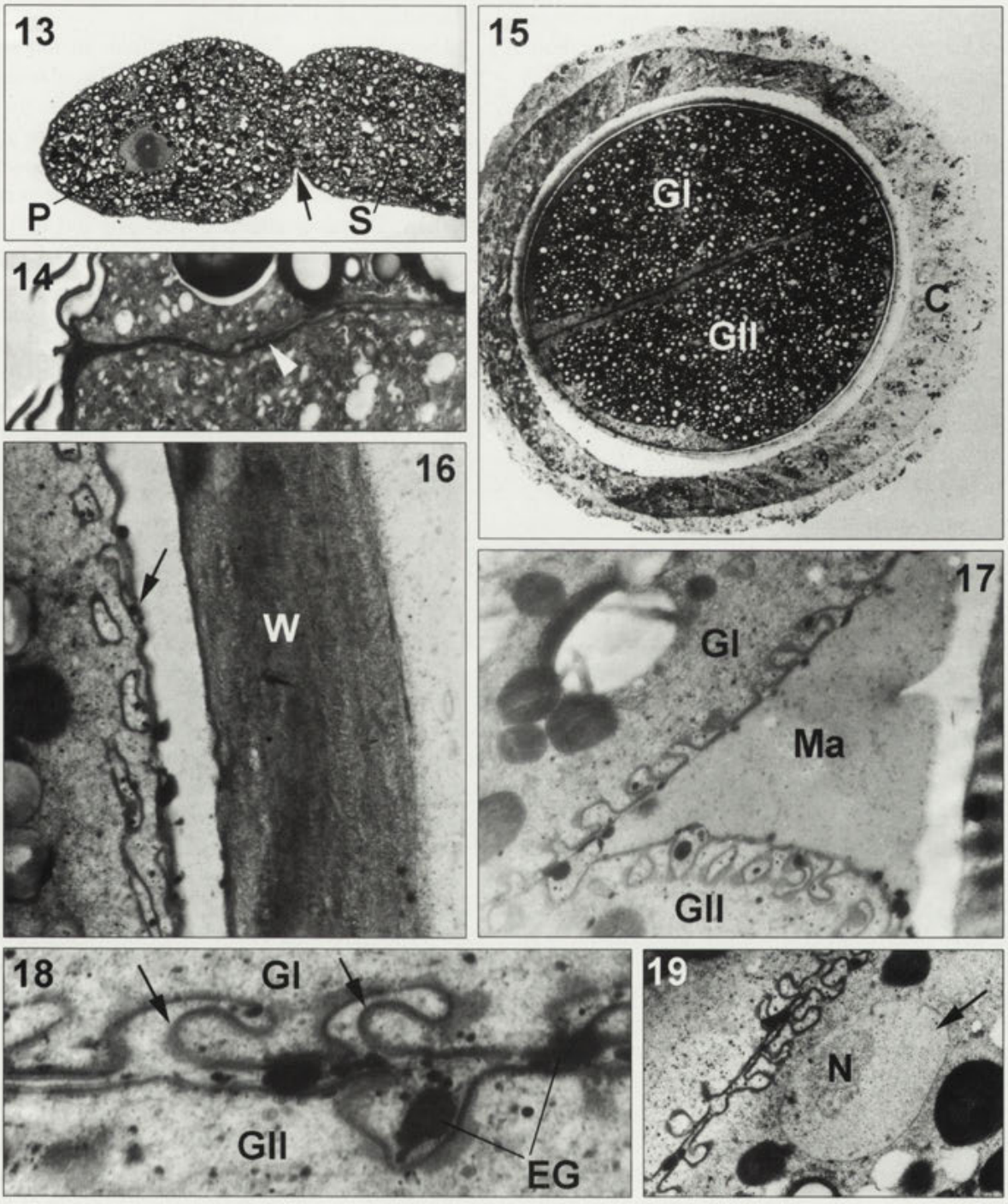
A single gametocyst has been found in the terminal part of the midgut (Fig. 15) of a specimen of *B. rhodani*. It is spherical (about 140 μm in diameter) and occupies almost the entire gut lumen. The gametocyst is uniformely enveloped by a multilayered wall of 1.8 μm in thickness (Fig. 16). A homogeneous matrix accumulates laterally to the joining gamonts (Fig. 17). The epicytes change their shape and tend to flatten in such a way that the ectocyte border facing the cyst wall loses its tipical folded organi-



Figs. 1-6. *Enterocystis racovitzai* from the midgut of *Baetis rhodani* under interpherence contrast microscope (1), SEM (2-4) and TEM (5,6). 1 - view of a syzygy. The nucleus (N) of the primite (P) and of the satellite (S) is visible together with the septum (arrow). Note the apical protuberance (arrowhead) and the lateral lobe (asterisk) (x170); 2 - syzygy showing the two gamonts in pairs. P - primite, S - satellite (x500); 3 - detail of the apical protuberance of the primite delimited by some grooves (arrows) (x1140); 4 - syzygy inside the midgut (M) (x280); 5 - intravacuole young gregarine (G) which protrudes towards the midgut lumen. BB - epithelial cell brush border, C - midgut cell, N - gregarine nucleus including nucleolus (Nu) (x6400); 6 - intracellular gregarine (G) showing epicyte folds (EF) among which electron-dense material (arrows) is present. C - midgut cell (x33500)



Figs. 7-12. Various morphological aspects of the syzygy of *Enterocystis racovitzai* from *Baetis rhodani* under SEM (7, 12) and TEM (8-11). 7 - surface of the syzygy constituted by the epicyte folds (x8800); 8 - sequence of epicyte folds (EF) emerging from the ectocyte (E). Note the remarkable amount of paraglycogen granules (PG) (x8700); 9 - accumulation of extracellular electron-dense material (arrows) between adjacent epicyte folds (EF).-The ectocyte cytoplasm shows electron-dense granules (EG), vacuoles (V) and fibrillar components (arrowheads) (x32500); 10 - detail of epicyte folds showing their multilayered border (arrow) with a striated organization in the apical portion (white arrowhead). Note the electron-dense granule (black arrowhead) adherent to the cytomembranes (x84000); 11 - cross section of the primite filled up by paraglycogen granules. N -nucleus. Note the scattered filaments (arrowheads) of the nucleolus (Nu) (x11500); 12 - decorticated gregarine showing the arrangement of the paraglycogen inclusions (x1600)



Figs. 13-19. TEM images of syzygy of *Enterocystis racovitzai* from *Baetis rhodani*, in the midgut lumen (13, 14) and in the cyst (15-19). 13 - contact region (arrow) between primite (P) and satellite (S) (x430); 14 - longitudinal section showing the parallel alignment (arrowhead) between the two confronting membranes (x7200); 15 - gametocyst occupying the entire gut lumen. C - epithelial cells of the midgut, GI-GII - gamonts in association (x450); 16 - multilayered organization of the wall (W) of the gametocyst. Remodelling of the cell surface facing the cyst wall leads to epicyte fold disappearance (arrow) (x9200); 17 - homogeneous matrix (Ma) accumulation between gamonts (GI-GII) (x20500); 18 - epicyte fold shape (arrows) along the contact surface of two gamonts (GI-GII) and the accumulation of electron-dense granules (EG) along the facing pairs (x33500); 19 - gamete in formation (arrow). N - nucleus (x8000)

zation (Fig. 16). On the contrary, along the contact zone of the two joined gamonts a thin space becomes evident and delimited by a series of irregularly folded projections. Dense granules accumulate in this interspace (Fig. 18). Gametes in formation can be seen as spherical cells (2,5-3 μm in diameter) overlapped by paraglycogen granules that make them hardly visible (Fig. 19).

DISCUSSION

Particular attention has been paid to the association between the aquatic developmental phases of various aquatic insect groups and gregarines (in addition to those reported in the introduction section: Baudoin 1967, Moretti and Sorcetti Corallini 1976, Percival *et al.* 1995, Sarkar 1995). These studies represent a significant contribution to our knowledge about host/parasite relationship, gregarine diversity and distribution. In particular, the species-specific association raises question about transmission mechanism and coevolution between host and parasite.

According to previous observations (Codreanu 1940; Desportes 1963, 1966; Geus 1969), the present study on *E. racovitzai* confirms that reproductive pairs of the parasite are the most common developmental phase found in the midgut of the nymphs of the mayfly *B. rhodani*. The partners show an apical/basal polarity and their morphological sexual dimorphism increases along with their level of maturation. The fine organization of *E. racovitzai* conforms broadly to that of other gregarines mainly as for the epicyte structure as for the large amount of paraglycogen that may be utilized during the successive stages of the parasite development (Vegni Talluri and Dallai 1985). Extrusion of electron-dense material through the epicytes as mucopolysaccharide granules has been observed in *E. fungoides* (Desportes 1974). Similar inclusions are present among differentiating epicytes of *E. racovitzai* when the gregarine is still endocellular and can be observed during the developmental steps of the parasite. Indeed, electron-dense granules are included among the modified epicytes at the interface between the reproductive pairs of the cyst and beneath the cyst wall. We identify these granules as mucopolysaccharides on the basis of the resemblance of our images with those of authors who used histochemical tests. We reckon that this material is in some manner associated with the phases of the life cycle of the parasite, being involved in the gliding movement, in the formation of the homogeneous matrix between the gamonts in the cyst, and in the cyst wall differentiation. Indeed, it seems acceptable that some granules may contribute to build the future wall, as reported in monocystid gregarines (Martinucci and Crespi 1979).

The relevance of the epicyte folds in the secretory process has been repeatedly stressed also in reference to gregarine gliding movement (Dallai and Vegni Talluri 1983, Vegni Talluri and Dallai 1983). Gregarine gliding is a complex phenomenon in which the supposed acto-myosin mechanism (King *et al.* 1982) has been corroborated by the occurrence of actin-like and myosin-like proteins in the cell cortex (Ghazali *et al.* 1989, Ghazali and Schrével 1993).

As observed by Desportes (1974) in *E. fungoides*, also in *E. racovitzai* a gradual involution of the epicyte folds between associated gamonts takes place. In *Gregarina polymorpha* cells in syzygy keep in contact by means of septate-like junctions allowing firm membrane adhesion (Dallai and Vegni Talluri 1988). A comparison between the contact zone of the two confronting gamonts of *E. racovitzai* living both in the lumen gut and inside the cyst, showed that an intercellular space becomes evident in this last developmental stage only. This feature suggests that a tight adhesion occurs mainly in the syzygy not protected by the cyst wall.

The intensity of parasitism is extremely high and in some specimens the whole gut is filled up with syzygies. In particular, the single cyst tends to occupy almost entirely the lumen of the terminal region of the midgut. Such a remarkable number of gregarines has to affect the food movement and the assimilation in the insect gut as demonstrated by Lipa (1967) and by Brooks and Jackson (1990). These results are in contrasts with the belief that parasites do not interfere with the metabolic processes of their hosts (Lipa *et al.* 1996). No evident damage has been detected in the specimens of *B. rhodani* examined in the present study. The co-existence of the parasite both as differentiating gregarines inside the cells of the midgut and in pairs in the lumen supports the notion that the insects could be infected in different moments in the course of their aquatic growth.

Nevertheless, it is worth stressing that the presence of the parasite in its final developmental phase is concomitant with the conclusion of the aquatic life-cycle of the host.

Bobyleva (1963, in Arvy and Peters 1973) elucidated the life-cycle of *E. ensis* showing that further gametocyst development takes place after cyst deposition in water. In contrast, in the cyst of *E. racovitzai* some identical gametes in formation were observed in both sexually complementary partners.

In conclusion, this investigation on *E. racovitzai* provides additional data on the fine morphology of gregarines

harboured in mayflies. This report is the first finding of gregarines associated to Italian Ephemeroptera, thereby contributing to expand knowledge on Enterocystidae, a parasitic group exclusively associated to mayflies.

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ACTA
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Interstitial Testate Amoebae (Rhizopoda: Arcellinida and Gromida) from the Finnish Coast of the Baltic Sea and Summary Check List of the Interstitial Testate Amoebae in the Baltic Sea

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Summary. As a result of the present study of the interstitial testate amoebae from the Finnish Coast of the Baltic Sea a total of 34 interstitial testate amoebae have been found, 21 of which are psammobiotic and 13 are psammophilic and psammoxenic. A review on the interstitial testate amoebae in the Baltic Sea, known up to now, has been made. The total check list of the Baltic interstitial testate amoebae includes 53 taxa, 21 of which are psammobiotic (40 %), 12 are psammophilic (23 %) and 20 are psammoxenic (37 %). The high percentage of the psammophilic and psammoxenic testate amoebae is due to the very low salinity of the Baltic Sea as compared with typical seas and oceans.

Key words: Baltic Sea, Finnish Coast, interstitial, psammon, testate amoebae.

INTRODUCTION

The interstitial testate amoebae inhabiting the littoral sandy beaches of the Baltic Sea coasts have been quite insufficiently studied so far. The first investigation on this specific taxocenose was accomplished on the Polish Coast of the Baltic Sea (Golemansky 1970a, 1973). Despite the expectations of a rare occurrence of psammobiotic testaceans in the littoral psammal of the Baltic Sea because of its low salinity, a total of 20 testate amoebae was found in the above-mentioned research. A considerable proportion of them - 12 species were strict psammobionts or psammobiotics. Four new species of testate amoebae were described from the littoral psammal of the Baltic Sea (*Difflugiella psammophila* Gol., *Micropsammella retorta* Gol., *Psammonobiotus balticus* Gol. and *Cyphoderia littoralis* Gol.) which were found later in other seas and oceans as well. Then, in samples from the Russian and Estonian Coast of the Gulf of Finland other interstitial testate amoebae were found and the list of Testaceans described from the sandy littoral of the Baltic Sea increased to 35 species (Golemansky 1983). Due to the low salinity in the underground waters of the sandy beaches of the Baltic Sea it was also established that psammoxenic and psammophilic testate amoebae prevailed (23 species and varieties), while only 12 psammobiotic species were found.

In 1984, through a scientific collaboration between the Bulgarian and the Finnish Academies of Sciences we visited many sandy beaches on the Finnish Coast of the Baltic Sea and collected a rich material of interstitial testaceans. The results of the studies of this collection are

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the object of the present work, which broadens and completes our knowledge of the interstitial testate amoebae of the Baltic Sea.

MATERIALS AND METHODS

The materials for the present study were collected in the period of 21-29 May, 1984 from 8 sandy beaches and 18 stations on the Finnish Coast of the Baltic Sea. The following sandy beaches and stations were explored in greater detail:

A. Sando Island, Nauvo Seili, 21.05.1984. South sandy beach, about 500 x 200 m.
St. 1. Distance from sea water (D): 2 m; depth in the sand (P): 0.20 m.; salinity of water (S): 2.8 ‰.
St. 2. D: 4 m; P: 0.40 m; S: 2.0 ‰.
St. 3. D: 5-6 m; P: 0.60 m; S: 0.6 ‰.

B. Knaplo Island, 21.05.1984. Little sand-gravel beach.
St. 1. D: 2 m; P: 0.25 m; S: 3.2 ‰.

C. Auland Island, Degersand, 27.05.1984. Homogeneous, well developped sandy beach, about 300 x 20 m.
St. 1. D: 1 m; P: 0.15 m; S: 3.0 ‰.
St. 2. D: 3-4 m; P: 0.45 m; S: 1.8 ‰.
St. 3. D: 10 m; P: 0.60 m; S: 0.9 ‰.

D. Tvarmine, Zoological station, 28.05.1984. Homogeneous sandy beach.
St. 1. D: 6 m; P: 0.45 m; S: 3.3 ‰.
St. 2. D: 11 m; P: 0.85 m; S: 1.3 ‰.

E. Tvarmine, Tullinemi beach, 28.05.1984. Heterogeneous sand-gravel beach.
St. 1. D: 1 m; P: 0.25 m; S: 4.3 ‰.
St. 2. D: 3 m; P: 0.65 m; S 2.2 ‰.

F. Hanko, House of the four winds, 29.05.1984. Homogeneous sandy beach.
St. 1. D: 3-4 m; P: 0.60 m; S: 3.4 ‰.

G. Henriksbeig, Hogholmen, 29.05.1984. Large heterogeneous sandy beach.
St. 1. D: 1-2 m; P: 0.10 m; S: 3.8 ‰.
St. 2. D: 15-20 m; P: 0.20 m; S: 1.2 ‰
St. 3. D: 35-40 m; P: 0.75 m; S: 1.1 ‰.

H. Lappvik sandy beach, 29.05.1984.
St. 1. D: 1 m; P: 0.15 m; S: 3.8 ‰.
St. 2. D: 3 m; P: 0.35 m; S: 2.0 ‰.
St. 3. D: 5 m; P: 0.55 m; S: 1.2 ‰.

The different width and inclination of the investigated sandy beaches permitted us to take samples from some stations situated on cross profiles from the sea to the dry land and standing at various distances from the water-line. Up to 3 cross-situated stations were investigated on well-developed sandy beaches, while only one or two stations were explored on steep or narrow sandy bands. Samples from the supralittoral sandy band are taken after holes had been dug according to the Chappuis method (1942). About 100 cm^3 of underground water and sand (1:1) were taken from every studied station for laboratory investigation. The collected underground water was not filtered through a hydrobiological net, because a considerable part of the interstitial testate amoebae have cross-sections of less than 20 μm and pass easily through the openings even in the finest hydrobiological nets.

The collected samples were kept alive until they were tested for salinity in laboratory. The halogen ions were analysed by argentometric titration and the salinity was calculated by Knudsen formula S ‰ = 0.030 + 1.8050 Cl ‰ (Strickland and Parson 1965). For a longer period of investigation the samples were kept fixed by 2% neutral formaldehyde.

RESULTS

The laboratory investigation of the collected material shows that the interstitial testacean fauna in the sandy beaches of the Finnish Coast of the Baltic Sea is relatively rich and highly varied. A total of 34 testate amoebae belonging to 18 genera has been established in the explored stations. The list of the testate amoebae found and their distribution in the investigated sandy beaches and stations are given in Table 1.

It is evident from Table 1 that the interstitial testacean fauna from the Finnish Coast of the Baltic Sea consists of a relatively high number of taxa, belonging to different ecological categories. The number of strict psammobionts is highest (21 species). The number of psammophilic and psammoxenic testate amoebae living mostly in the fresh water basins is also comparatively high (13 species). Compared to the seas and oceans with normal salinity, where the psammophilic and psammoxenic testaceans comprise about 30 % of the interstitial testacean fauna, the percentage of psammophilic and psammoxenic amoebae in the studied stations on the Finnish Coast of the Baltic Sea is higher - 38 %. This fact is due to the close vicinity of the numerous fresh-water basins (lakes, swamps, rivers), to the investigated sandy bands and stations. The salinity of the underground waters is rather low - less than 4.5 ‰. It is known that the salinity limiting the development of fresh-water testate amoebae is 5 ‰ (Dehtjar 1969).

CONCLUSIONS

The former investigations on the interstitial testate amoebae of the Baltic Sea cover only its South-Eastern coast (Golemansky 1970a, 1973, 1983). As a result of the

Table 1. Interstitial testate amoebae from the Finnish Coast of the Baltic Sea. A-Sando Island, Nauvo Seili, 21.05.1984, B-Knaplo Island, 21.05.1984, C-Auland Island, Degersand, 27.05.1984, D-Tvarmine, Univ. station, 28.05.1984, E-Tvarmine, Tullinemi beach, 28.05.1984, F-Hanko, 29.05.1984, G-Henriksberg, Hogholmen, 29.05.1984, H-Lappvik beach, 29.05.1984, Ec-ecological category

Taxa	A			B	C			D		E		F	G			H			Ec
Stations	1	2	3	1	1	2	3	1	2	1	2	1	1	2	3	1	2	3	
Arcellinida Kent																			
Arcellidae Ehrenberg																			
Arcella hemisphaerica Perty													+						PX
A. rotundata Playfair													+	+	+				PX
Microcoryciidae De Saedeleer																			
Microchlamys patella (Clap.& Lach.)	+				+	+						+	+	+	+	+	+		PF
Centropyxidae Deflandre																			
Centropyxis aculeata (Ehrenb.)								+						+	+				PX
C. aculeata v. oblonga Defl.																+			PX
C. cassis v. spinifera Defl.													+						PX
C. constricta (Ehrenb.)													+	+		+			PX
C. minuta Defl.														+					PX
Centropyxiella arenaria Valk.					+			+		+			+			+	+		PB
C. golemanskyi Chardez								+											PB
Difflugiidae Awerintzev																			
Difflugia lucida Pen.		+															+		PF
Difflugia sp.						+													PX
Cryptodifflugiidae Rhumbler																			
Cryptodifflugia lanceolata Gol.					+	+					+	+							PB
Difflugiella psammophila Gol.						+						+						+	PB
Gromiida Clap. & Lach.																			
Psammonobiotidae Gol.																			
Psammonobiotus communis Gol.	+	+			+	+		+		+	+	+				+	+	+	PB
P. golemanskyi Chardez					+														PB
P. linearis Gol.										+	+					+	+		PB
Pseudocorythion acutum (Wailes)	+	+	+							+		+					+	+	PB
P. wailesi Gol.	+																		PB
Corythionella acolla Gol.					+		+			+		+				+	+		PB
C. minima Gol.	+																	+	PB
Micramphora pontica Valk.											+								PB
Messemvriella filosa Gol.												+							PB
Micropsammella retorta Gol.												+					+		PB
Ogdeniella elegans (Gol.)																	+		PB
Cyphoderiidae De Saedeleer																			
Cyphoderia ampulla (Ehrenb.)	+	+								+	+	+				+			PF
C. compressa Gol.												+				+			PB
Euglyphidae Wallich																			
Euglypha laevis Perty						+	+		+										PB
E. rotunda Wailles-Pen.		+		+		+	+					+							PB
Trinema complanatum Pen.		+					+		+										PB
T. enchelys (Ehrenb.)						+		+											PB
T. lineare Pen.						+		+	+								+		PB
Pseudodifflugiidae Schlumb.																			
Pseudodifflugia fascicularis Pen.												+							PF
Pseudodifflugia sp.			+																PF?
Total	6	6	2	1	6	9	4	6	3	6	5	12	6	5	3	9	10	4	34

Abbreviations: PB - psammobiotic, PF - psammophilic, PX - psammoxenic

Table 2. Check list and distribution of the interstitial testate amoebae in the Baltic Sea

Taxa	Polish coast (Golemansky 1970, 1973)	Estonian coast (Golemansky 1983)	Russian coast (Golemansky 1983)	Finnish Coast (Present study)	Ecological category
Arcellinida Kent					
Arcella hemisphaerica Perty				+	PX
A. rotundata Playfair				+	PX
A. vulgaris Ehrenb.			+		PX
Microchlamys patella (Clap.& Lach.)	+	+	+	+	PF
Centropyxis aculeata (Ehrenb.)			+	+	PX
C. aculeata v. oblonga Defl.				+	PX
C. aerophila Defl.			+		PX
C. cassis v. spinifera Defl.				+	PX
C. constricta (Ehrenb.)			+	+	PX
C. minuta Defl.				+	PX
Cyclopyxis kahli Defl.	+				PX
Cyclopyxis sp.		+	+		PX?
Centropyxiella arenaria Valk.	+	+		+	PB
C. golemanskyi Chardez				+	PB
Difflugia lucida Pen.	+	+	+	+	PF
D. linearis (Pen.)		+			PF
Difflugia sp.				+	PX?
Difflugiella psammophila Gol.	+			+	PB
Hyalosphaenia cuneata Stein	+	+			PF
Cryptodifflugia lanceolata Gol.	+	+		+	PB
Pommoriella valkanovi Gol.		+			PB
Gromiida Clap.& Lach.					
Assulina muscorum Greef			+		PX
Cyphoderia ampulla (Ehrenb.)	+	+	+	+	PF
C. compressa Gol.				+	PB
C. littoralis Gol.	+				PB
Corythionella acolla Gol.	+	+		+	PB
C. minima Gol.		+		+	PB
C. sudzuki Chardez		+			PB
Pseudocorythion acutum (Wailes)	+	+		+	PB
P. wailesi Gol.				+	PB
Psammonobiotus communis Gol.	+	+	+	+	PB
P. linearis Gol.	+	+		+	PB
P. minutus Gol.		+			PB
P. golemanskyi Chardez				+	PB
Messemvriella filosa Gol.				+	PB
Micramphora pontica Valk.	+			+	PB
Micropsammella retorta Gol.	+	+		+	PB
Ogdeniella elegans (Gol.)	+			+	PB
Ogdeniella sp.	+				PB
Euglypha laevis Perty		+	+	+	PF
E. compressa Carter		+			PX
E. cristata Leidy			+		PX
E. filifera Pen.			+		PX
E. rotunda Wailles-Pen.	+	+	+	+	PF
E. tuberculata (Duj.)			+		PX
Euglypha sp.		+	+		PX
Tracheleuglypha dentata (Vejd.)		+			PX
Trinema complanatum Pen.		+	+	+	PX
T. enchelys (Ehrenb.)			+	+	PF
T. lineare Pen.			+	+	PF
Pseudodifflugia fascicularis Pen.	+			+	PF
P. c. f. gracilis Schlumb.			+		PF?
Pseudodifflugia sp.	+		+	+	PF?
Total	20	23	21	34	53

Abbrevations: PB - psammobiotic; PF - psammophilic; PX - psammoxenic

previous and the present studies a total of 53 testate amoebae has been established in the underground waters of the sandy supralittoral part of the Baltic Sea (Table 2). The total number of the psammobiotics found up to now is 21 species. The cosmopolite *Psammobiotus communis* is especially wide-spread and found in all studied regions of the Baltic Sea. It is known to be a euryhaline inhabitant found twice in some European refreshed lakes, remains of the pliocene Sarmatian Sea (Golemansky 1970b, 1994). Very common strict psammobionts in the coastal underground waters of the Baltic Sea are the following six species: *Pseudocorythion acutum, Corythionella acolla*, *Micropsammella retorta, Centropyxiella arenaria, Cryptodifflugia lanceolata* and *Psammobiotus linearis*. They have been established in many stations on the Finnish Coast and also belong to the category of euryhaline psammobionts. It has been interesting to find from an ecological point of view *Centropyxiella golemanskyi* in the sandy beach of Tvarmine Zoological station at salinity of about 3‰ (Table 1). So far this species has been known only in the supralittoral psammal of the North Sea and in the sublittoral psammal of the Atlantic Ocean at a very high salinity (Chardez 1977, Golemansky 1991).

It is also evident from Table 2 that the total number of psammophilic and especially psammoxenic testate amoebae in the coastal underground waters of the Baltic sandy beaches is higher compared to the seas with normal salinity. In the Mediterranean basin for example, 57 interstitial Testacean species have been found so far, 29 of which (51 %) are psammobiotic, 10 (18 %) are psammophilic and 18 (32 %) are psammoxenic (Golemansky 1990). As it is shown in Table 2, these data for the Baltic Sea are as follows: psammobiotic - 21 species (40 %), psammophilic - 12 (23 %) and psammoxenic - 20 (37 %). Undoubtedly this fact is due to the relatively lower salinity of the Baltic Sea and of coastal underground waters in comparison with the seas and oceans with normal salinity.

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Interstitial Testate Amoebae (Rhizopoda: Testacea) from the Italian Coast of the Mediterranean Sea

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Summary. As a result of the present study, 23 interstitial testate amoebae from the southern Italian Coast of the Mediterranean Sea have been recorded. The sand beaches composed of homogeneous fine sand are mainly rich in psammobiotic testate amoebae. A review of investigations on the Italian Coast carried out so far shows that a total of 31 interstitial testate amoebae have been found, 25 of which are psammobiotic and 6 of which are psammophilic and psammoxenic. The low percentage of the psammophilic and psammoxenic testaceans is due to the high salinity of the underground supralittoral waters and to the absence of considerable freshwater lakes, rivers and marsh-lands near the investigated sand beaches.

Key words: interstitial, Italian Coast, Mediterranean Sea, psammon, testate amoebae.

INTRODUCTION

The interstitial testate amoebae, living in the underground waters of marine sandy beaches, are common components of the marine microzoobenthos and represent a specific taxocenose. All testate amoebae which only live in the underground waters of the marine supralittoral are considered as psammobiotic (PB) (Golemansky 1980, 1994). The psammophilic testate amoebae (PF) live and breed in the underground waters of marine sand beaches, but they also inhabit freshwaters, moss and soil. Psammoxenic testate amoebae (PX) usually only live in freshwaters, soil and moss. They are occasionally carried in the sand supralittoral biotops by continental underground waters. This taxocenose, with its' three ecological categories, is composed of more than 80 different taxa. It has a cosmopolitan distribution in marine sandy supralittoral waters (Golemansky 1980, 1990).

Investigations on the interstitial testate amoebae from the Italian Coast of the Mediterranean Sea are scanty and fragmentary. In my first article on the Rhizopods and the Heliozoans from the Mediterranean supralittoral waters I reported a total of 21 species of testaceans, 11 of which were found along the Italian Coast, namely from Lago Lungo to Kiozga (Golemansky 1976). Several years later, Chardez (1989) reported the presence of 10 psammobiotic testate amoebae collected from the regions of Riccione and Porto Verde in the Adriatic Italian Coast. In a critical review on the interstitial testate amoebae from the Mediter-

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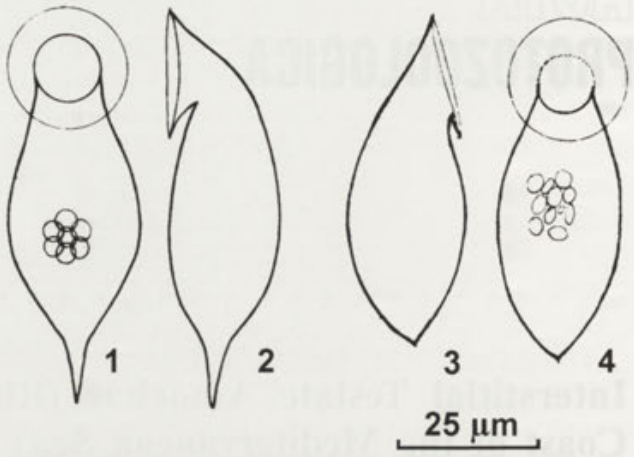
ranean basin I found (Golemansky 1990) that the composition of the total of 57 known testaceans in the Mediterranean Sea was: 29 psammobiotic, 10 psammophilic and 18 psammoxenic. Only 17 interstitial testate amoebae were known from the Italian Coast at this time.

I visited 4 new localities on the Italian Coast of the Mediterranean Sea and collected some new samples from well developed sandy beaches in 1987 and 1991. In the present paper I present the results of my investigations, trying to make a survey of the present knowledge of the interstitial testate amoebae from the Italian Coast of the Mediterranean Sea.



Figs. 1-4. *Pseudocorythion acutum* (Wailes). 1, 2 - typical form from the Black Sea. 1 - ventral view; 2- lateral view. 3, 4 - acaudal specimen from the sand beach of Iscchia Island (the Mediterranean Sea)

MATERIALS AND METHODS

The materials for the present investigation were collected from 6 places in the southern part of the Italian Coast, as follows:

Sorento, Capo di Sorento, 16 May, 1987. Sand black, heterogeneous.
Station (St.) 1. Distance from the sea (D): 1 m.; depth in the sand (P): 0.20 m.; salinity of the underground water (S): 34 ‰.
St. 2. D: 2 m.; P: 0.30 m.; S: 33 ‰.
St. 3. D: 4 m.; P: 0.35 m.; S: 33 ‰.

Capri Island, Central beach, 17 May, 1987. Sand heterogeneous, with gravel.
St. 1. D: 3 m.; P: 0.35 m.; S: 35.6 ‰.

Iscchia Island, St. Pietro beach, 18 May, 1987. Sand homogeneous, black, finely granulated.
St. 1. D: 1 m.; P: 0,15 m.; S: 35 ‰.
St. 2. D: 4 m.; P: 0,35 m.; S: 32 ‰.
St. 3. D: 8 m.; P: 0,60 m.; S: 28,5 ‰.

Iscchia Island, Pescatori beach, 18 May, 1987. Sand heterogeneous.
St. 1. D: 3 m.; P: 0,25 m.; S: 34,5 ‰.
St. 2. D: 6 m.; P: 0,45 m.; S: 32,5 ‰.

Iscchia Island, Chiaia beach, 21 May, 1987. Sand heterogeneous.
St. 1. D: 2 m.; P: 0,25 m.; S: 33,5 ‰.

Pesaro, Central beach, 25 May, 1991. Sand homogeneous, finely granulated.
St. 1. D: 2 m.; P: 0,20 m.; S: 34,8 ‰.
St. 2. D: 3 m.; P: 0,40 m.; S: 34,5 ‰.
St. 3. D: 5 m.; P: 0,60 m.; S: 32 ‰.

RESULTS

A total of 23 interstitial testate amoebae have been observed in the studied sandy beaches (Table 1) as a result of the present investigation. The analysis of the results shows that the sandy beaches composed of homogeneous sands are well settled by interstitial testate amoebae. I found 12 species in the well developed sandy beach of St. Pietro at Iscchia and 11 in the Central beach at Pesaro. Both beaches are formed of homogeneous, finely granulated sands. Contrariwise, the heterogeneous sandy beaches with gravel, such as the beaches of Capri and Chiaia (Iscchia), are quite poor in interstitial testaceans.

The prevalent number of established interstitial testate amoebae are psammobiotic testaceans because of the high salinity of the underground waters in the investigated stations. Only three psammophilic testaceans, *Trinema lineare, T. enchelis* and *Euglypha rotunda,* were observed in the sandy beaches of Iscchia Island. This is probably due to the varying salinity of the underground waters or the proximity of fresh water near the investigated stations.

The major number of the studied psammobiotic testaceans are composed of typical forms, corresponding in their structure and morphometry to the original descriptions (Golemansky 1980). Below I will give some morphological information only for the species showing variability or deviations in comparison to the previous descriptions.

Pseudocorythion acutum (Wailes, 1927), Figs. 1-4

A strict psammobiont, quite common in the investigated seas. From the Mediterranean basin it is known in the coasts of Italy, Tunisia, Spain and France (Decloître 1972, 1975; Golemansky 1976, 1990; Chardez 1977, 1989). The typical forms are elongated, plagiostomic,

with a cleanly visible test structure (Figs. 1, 2). The dimensions of the shell vary from 60 to 80 μm. in length and from 20 to 28 μm. in width. The pseudostome is round, about 16 μm., with an enlargement around it and a diameter which is equal to the test diameter. There is a small spine at the posterior end of the test.

At all the studied stations along the St. Pietro beach (Iscchia) I observed a population of *P. acutum*, composed of small specimens with atypical structure of the test (Figs. 3, 4). They have an elongated test with a round cross section, but the caudal spine is reduced or it is missing. The test is formed by circular or slightly elliptic idiosomes. In some specimens the idiosomes are not cleanly visible or their number is reduced and the structure of the test looks like the structure of *Corythionella* (Golemansky, 1980). The dimensions of the observed forms were of 46-55 μm in length and 17-21 μm in diameter.

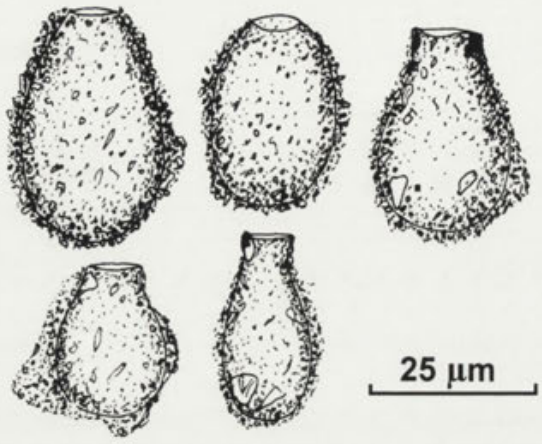


Fig. 5. Five different specimens of *Allogromia* sp. from the sand beach of Sorento (the Mediterranean Sea)

Table 1. A check list and distribution of the interstitial testate amoebae in the investigated sand beaches

Taxa	Sorento 16.05.1987			Capri 17.05.1987	Iscchia St. Pietro 18.05.1987			Iscchia Pescatori 18.05.1987		Iscchia Chiaia 21.05.1987	Pesaro 25.05.1991		
	Stations												
	1	2	3	1	1	2	3	1	2	1	1	2	3
Psammonobiotus communis Gol.	+		+			+	+	+		+		+	
P. minutus Gol.						+							
P. golemanskyi Chardez	+					+				+			
Pseudocorythion acutum (Wailes)					+	+	+	+					
Corythionella acolla Gol.					+	+						+	
C. minima Gol.						+							
Messemvriella filosa Gol.							+						
Micramphora pontica Valk.												+	
Micropsammella retorta Gol.												+	
Centropyxiella arenaria Valk.												+	
Ogdeniella elegans (Gol.)									+				
O. lucida (Gol.)		+				+					+	+	
O. taschevi (Gol.)					+							+	
Cyphoderia littoralis Gol.						+							+
Campascus interstitialis Gol.	+		+	+	+	+							
Cryptodifflugia lanceolata Gol.		+				+	+		+				
Allogromia sp.	+	+				+							
Rhabdogromia flexilis (Hogl.)												+	
Lagenidiopsis valkanovi Gol.				+		+	+	+				+	
L. elegans (Gruber)				+								+	
Trinema lineare Pen.								+					
T. enchelys Pen.							+	+					
Euglypha rotunda Pen.								+					
Total	4	3	2	3	4	12	6	6	2	2	1	10	1

Table 2. A check list and distribution of the intestitial testate amoebae on the Italian Coast of the Mediterranean Sea

Taxa	Localities	References	Ecological category
Psammonobiotus balticus Gol.	Riccione	Chardez (1989)	PB
P. communis Gol.	Lago Lungo, Kjozga, Sorento, Iscchia, Pesaro, Riccione	Golemansky (1976, 1990, present study),Chardez (1989)	PB
P. golemanskyi Chardez	Riccione, Sorento, Iscchia	Chardez (1989), present study	PB
P. minutus Gol.	Riccione, Iscchia	Chardez (1989), present study	PB
Pseudocorythion acutum (Wailes)	Lago Lungo, Iscchia	Golemansky (1976, 1990, present study)	PB
P. wailesi Gol.	Riccione	Chardez (1989)	PB
Corythionella acolla Gol.	Kjozga, Iscchia, Pesaro, Riccione	Golemansky (1976, 1990, present study), Chardez (1989)	PB
C. minima Gol.	Kjozga, Lago Lungo, Iscchia	Golemansky (1976, 1990, present study)	PB
Ogdeniella elegans (Gol.)	Lago Lungo, Iscchia	Golemansky (1976, 1990, present study)	PB
O. lucida (Gol.)	Sorento, Iscchia, Pesaro	present study	PB
O. taschevi (Gol.)	Iscchia, Pesaro	present study	PB
Messemvriella filosa Gol.	Iscchia	present study	PB
Micropsammella retorta Gol.	Riccione, Pesaro	Chardez (1989), present study	PB
Micramphora atlantica Chardez & Thomas	Riccione, Pesaro	Chardez (1989), present study	PB
M. pontica Valk.	Riccione, Pesaro	Chardez (1976), present study	PB
Centropyxiella arenaria Valk.	Pesaro	present study	PB
Cyphoderia littoralis Gol.	Kjozga, Lago Lungo, Pesaro, St. Pietro	Golemansky (1976, 1990, present study)	PB
Campascus interstitialis Gol.	Riccione, Sorento, Capri, Iscchia	Chardez (1989), present study	PB
Cryptodifflugia lanceolata Gol.	Lago Lungo, Sorento, Iscchia	Golemansky (1976, 1990, present study)	PB
Difflugiella psammophila Gol.	Kjozga	Golemansky (1976, 1990)	PB
Rhabdogromia flexilis (Hoglung)	Pesaro	present study	PB
Lagenidiopsis elegans (Gruber)	Capri	present study	PB
L. valkanovi Gol.	Lago Lungo, Capri, St. Pietro	Golemansky (1976, 1990, present study)	PB
Allogromia sp.	Sorento, Iscchia, Pesaro	present study	PB
Pseudodifflugia sp.	Lago Lungo	Golemansky (1976, 1990, present study)	PB?
Hyalosphaenia cuneata Stein	Lago Lungo	Golemansky (1976, 1990)	PF
Microchlamys patella Clap. & Lach.	Lago Lungo	Golemansky (1976, 1990)	PF
Euglypha rotunda Pen.	Iscchia	present study	PF
Trinema enchelys Pen.	Iscchia	present study	PF
T. lineare Pen.	Iscchia	present study	PF
Phryganella sp.	Kjozga	Golemansky (1976, 1990)	PX?

Abbreviations: PB - psammobiotic; PF - psammophilic; PX - psammoxenic

It is known from the investigations of Chardez (1977), Chardez and Thomas (1980), Golemansky (1980) and Sudzuki (1979) that the test of *P. acutum* is quite variable depending on the ecological characteristics of the biotope such as salinity, sand granulometry, water dissolved oxygen etc. This must be taken into consideration by the taxonomists. So, I consider the observed population as one of the variations of *P. acutum* which is one of the most common and wide spread psammobiotic testate amoeba.

Allogromia sp., Fig. 5

In Sorento (Capo di Sorento, St. 1 and 2) and in Iscchia (St. Pietro, St. 2) I observed some specimens of testate amoebae with a characteristic structure and morphology.

of the test. The test is ovoid or pyriform, with a round cross section. The pseudostome of the anterior end is also circular. The test is formed of an organic basic membrane on which a great number of very small xenosomes and idiosomes are agglutinated. The structure of the test is similar to that of *Allogromia*. Sometimes the test wall is relatively thick because of a great number of particles agglutinated on it (Fig. 5).

The dimensions of the test vary from 22 to 33 μm in length with a diameter from 16 to 22 μm and a pseudostome of about 5-7 μm. The test of some specimens is slightly narrow at the anterior end and forms a brief neck around the pseudostome.

Living specimens were not observed.

CONCLUSION

As a result of the investigations carried on the Italian Coast of the Mediterranean Sea by Golemansky (1976), Chardez (1989) and the present study a total of 31 interstitial testate amoebae have been found so far (Table 2). The majority of them are strict psammobionts (25) and only 6 species are psammophilic or psammoxenic. The psammobionts *Psammonobiotus communis*, *Corythionella acolla*, *C. minima*, *Pseudocorythion acutum*, *Ogdeniella elegans*, *Campascus interstitialis* and *Cryptodifflugia lanceolata* are common and widely spread on the Italian Coast. They are eurybionts with a world-wide population in the seas and oceans (Chardez and Thomas 1980; Golemansky 1980, 1994).

In comparison with other intercontinental seas, such as the Black Sea and the Baltic Sea, the number of psammophilic and psammoxenic testate amoebae on the Italian Mediterranean Coast is very limited. This fact is due to the higher salinity of the underground supralitoral waters of the Mediterranean Sea on the one hand and to the absence of considerable fresh water biotops like continental lakes, rivers, etc. near the investigated sand beaches on the other hand.

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ACTA
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Biochemical and Spectroscopic Changes in Phycobiliproteins of the Cyanobacterium, *Aulosira fertilissima*, Induced by UV-B Radiation

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Summary. The effects of UV-B irradiation on the phycobiliproteins of the rice field cyanobacterium, *Aulosira fertilissima,* have been studied. Phycobiliproteins isolated and separated by sucrose density gradient centrifugation showed six fractions in the control which were reduced to three after 3 h of UV-B treatment. Except fraction 1, absorption spectra of all the UV-B treated fractions compared to corresponding fractions of the control showed a significant decline in the absorbence at all peaks. Fluorescence emission spectra of fraction 5 of the control sample showed an emission at 635 nm when excited at 620 nm indicating the presence of phycocyanin. The corresponding fraction 3 of the UV treated samples showed a decrease in the fluorescence emission indicating an impaired energy transfer from the accessory pigments to the photochemical reaction center. SDS-PAGE of the fractions showed a loss of high (linker polypeptides) and low ($\alpha\beta$ monomers) molecular mass proteins supportive of the interpretation of a disassembly of the accessory pigment complex due to exposure to UV-B.

Key words: *Aulosira*, cyanobacteria, phycobiliproteins, UV-B (280 – 315 nm) irradiation.

INTRODUCTION

Stratospheric ozone plays the critical role of filtering short wavelength radiation from the sun. In recent decades the increase of man made pollutants such as chlorinated fluorocarbons has resulted in gradual depletion of this gas layer with concomitant increases of UV-B reaching the surface of the Earth (Häder *et al.* 1995). This increased radiation is a stress factor to many organisms (Häder and Häder 1990, Gerber and Häder 1995, Sinha and Häder 1996) especially the microorganisms which have no epidermal covering.

Cyanobacteria are a unique group of prokaryotes which possess the ability of atmospheric nitrogen fixation using solar energy through photosynthesis and therefore act as potential contributors of soil nitrogen in a variety of ecosystems (Banerjee and Kumar 1992, Sinha and Häder 1996). This trophic independence of carbon and nitrogen gives them a great ability in maintaining crop productivity in many cases. In cyanobacteria, the primary light harvesting pigments are the water soluble phycobiliproteins which fall into three major classes, phycoerythrin, phycocyanin and allophycocyanin (Bryant 1991) and constitute about 40 - 50 % of the total cellular proteins (Sinha *et al.* 1995b). The phycobiliproteins are composed of ($\alpha\beta$) monomers

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covalently attached to a tetrapyrrole prosthetic group. The monomers form larger aggregates in the form of trimers and hexamers. These aggregates, in turn, are linked together by linker polypeptides forming the characteristic phycobilisomes of cyanobacteria and rhodophyceae (Glazer 1985).

UV-B radiation is known to damage photosynthetic pigments, and earlier investigations using absorption spectroscopy have shown that the accessory biliprotein pigments are bleached first followed by the carotenoids while the chlorophylls are damaged last (Sinha *et al.* 1995a) The present investigation aims at studying the effects of UV-B radiation on the phycobiliproteins of *Aulosira fertilissima,* a potent N_2-fixing cyanobacterium of Indian rice paddy fields using biochemical and spectroscopic analyses.

MATERIALS AND METHODS

Organism and culture conditions

Aulosira fertilissima was obtained from the Blue-Green Algal Culture Collection of I.A.R.I., New Delhi, India, grown in modified Chu 10 medium (Safferman and Morris 1964) and maintained in a culture room at 20° C and 12 W m^{-2} fluorescent light.

UV-B radiation source

For UV-B radiation studies exponentially growing cultures were harvested, transferred into sterile Petri dishes (25 mm in diameter) and exposed to artificial UV-B from a transilluminator (peak at 312 nm, Bachofer, Reutlingen, Germany). The radiation was about 5 W m^{-2} in the UV-B band determined using a double monochromator spectroradiometer (Optronics model 752, Orlando, FA, USA). The suspension was gently agitated by a magnetic stirrer during irradiation to warrant uniform exposure.

Spectroscopy

Absorption spectra were measured in 3 ml quartz cuvettes (2 mm thick) with an optical path length of 10 mm (Hellma, Müllheim, Germany) in a single beam spectrophotometer (DU 70, Beckman, Palo Alto, CA, USA). Samples taken during exposure were adjusted to the same protein content. Fluorescence emission spectra were measured with a spectrofluorimeter (RF 5000, Shimadzu, Kyoto, Japan).

Phycobiliprotein isolation

Phycobiliprotein isolation was performed as described earlier (Sinha *et al.* 1995b). Cultures were harvested and washed in 0.75 M phosphate buffer (pH 7.0) twice and resuspended in the same buffer and irradiated with UV. After 3 h of exposure aliquots were withdrawn and 1 mM PMSF solution and 2 mM EDTA were added. After this the cells were disrupted with a French press (Aminco Urbana IL, USA). To this suspension 1 % Triton-X and 5 % (w/v) sucrose was added and incubated at room temperature for 1 h. The suspension was then centrifuged at 40,000 x g at 15° C for 10 min. Phycobiliproteins were further separated by sucrose density gradient centrifugation (5 - 40 % w/v in 0.75 M phosphate buffer at pH 7.0). Aliquots of 1 ml were deposited on 11 ml volume of sucrose gradient and centrifuged for 16 h (15° C) at 150,000 x g in a swinging bucket rotor (SW 40, Beckman). The resulting fractions were retrieved by a syringe. Protein concentration was determined by the method of Bradford (1976).

SDS-PAGE

SDS-PAGE was carried out in a vertical system (2001, Pharmacia, LKB, Uppsala Sweden) with gels of 155 x 130 mm, 1.5 mm thick using the method of Laemmli (1970) with a gradient of 5 - 15 % T in the resolving gel. Gels were stained with Coomassie Brilliant Blue R 250 and dried in a gel dryer (Bio-Rad, Richmond, CA).

RESULTS

The crude pigment extract of *A. fertilissima* contained phycobiliproteins, carotenoids and chlorophyll *a* as indicated by the absorption spectra (data not shown). Five major peaks at 338, 434, 487, 620 and 668 nm were recorded. When irradiated with UV-B for 3 h the decline of the peaks appeared to be the highest for phycocyanin (620 nm) followed by the carotenoids (487 nm) whereas the reduction of the chlorophyll peaks (434 nm and 668 nm) was comparatively low. Sucrose density gradient centrifugation of non irradiated (control) samples showed six fractions while there were only three fractions in the irradiated samples. The absorption spectra of the different fractions of the control showed that the 1st, 2nd and 3rd fractions were almost similar and represented the major chlorophyll and carotenoid peaks (434, 487, and 668 nm) while fractions 4, 5 and 6 showed mainly phycocyanin absorption (620 nm). Figure 1 shows the absorption spectra of the 1st, 3rd and 5th fractions of the control. The absorption spectrum of fraction 2 was similar to that of fraction 1, and the absorption spectra of fractions 4 and 6 were similar to that of fraction 5 (data not shown). Figure 2 shows the absorption spectra of fractions of UV-B treated samples. The 1st and 2nd fractions contained mainly chlorophyll and the carotenoids (434, 487 and 668 nm) while the 3rd fraction showed phycocyanin absorption most of which had been bleached. The 2nd fraction which corresponds to the 3rd fraction of control is also very reduced. There was more or less no change in the absorption of fraction 1 of control and corresponding fraction 1 of UV-B treated samples.

Fluorescence excitation at 434 nm of fraction 1 of the control samples and fraction 1 of the UV-B treated

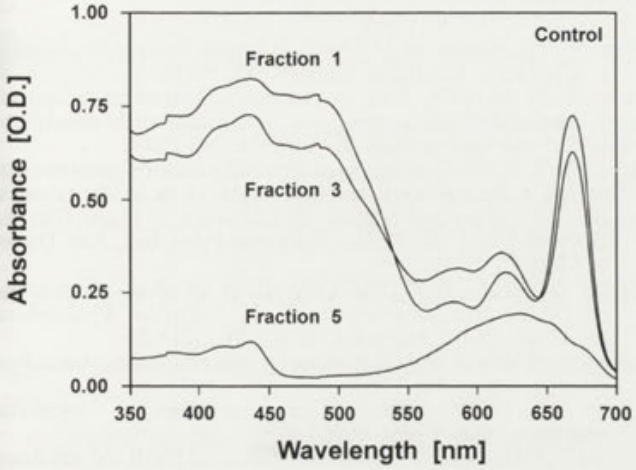


Fig. 1. Absorption spectra of sucrose density gradient fractions (1, 3 and 5) of non irradiated (control) *A. fertilissima*. For details see text

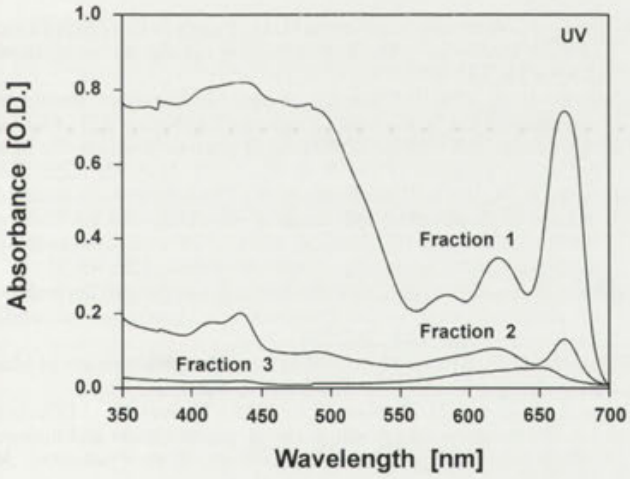
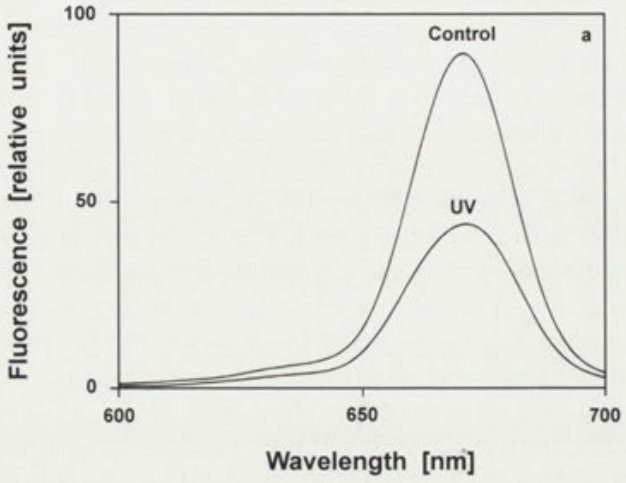


Fig. 2. Absorption spectra of sucrose density gradient fractions (1, 2 and 3) of 3 h UV-B treated *A. fertilissima*. For details see text



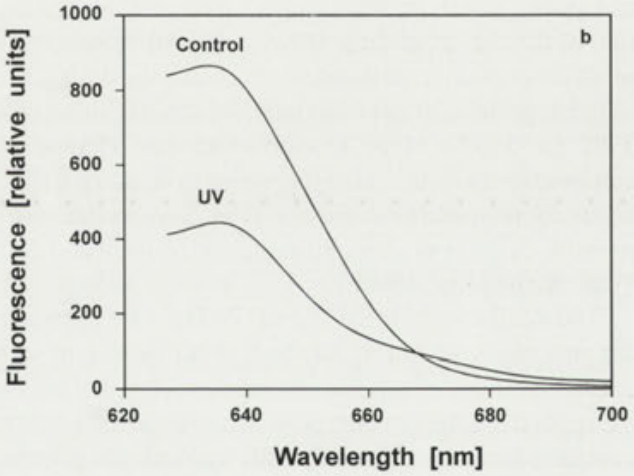


Fig. 3. Fluorescence emission spectra of fraction 1 of control samples and fraction 1 of UV-B treated samples of *A. fertilissima* when excited at 434 nm (a) and fraction 5 of control samples and corresponding fraction 3 of UV-B treated samples when excited at 620 nm (b), using a slit width of 5 nm

samples resulted in an emission maximum at 671 nm which declined with UV-B treatment (Fig. 3a). Fluorescence excitation at 620 nm of fraction 5 of the control samples and fraction 3 of the UV-B treated samples resulted in an emission maximum at around 635 nm which also declined after 3 h of UV-B treatment (Fig. 3b). The fluorescence of the 4th and the 6th fractions was similar to that of fraction 5 while fluorescence of fraction 2 and 3 was similar to that of fraction 1 (data not shown).

The unirradiated control fractions 1 and 2 showed faint bands with molecular masses of 18, 29 and 36 kDa (data not shown). While fraction 3 showed all the polypeptides between 14 to above 66 kDa; fractions 4, 5 and 6 mainly contained low molecular mass proteins between 16 to 22 kDa (α β monomers of phycocyanin) and some faint bands between 24 to 45 kDa (rod-linker polypeptides) (data not shown). When irradiated with UV-B for 3 h there was a loss in the intensity of the bands between 16 to 22 kDa and between 24 - 45 kDa (data not shown).

DISCUSSION

The results of the present investigation show that UV-B causes a marked effect on the phycobiliproteins of *A. fertilissima*. The changes in the absorption and the

fluorescence spectra as well as SDS-PAGE analysis of the phycobiliproteins indicate a loss in high molecular weight linkers and low molecular weight α β monomers. This damage results in a decreased energy transfer from phycobiliproteins to the photosystem due to exposure to UV radiation. Similar results have been reported previously (Fischer and Häder 1992; Sinha *et al.* 1995b,c, 1997; Lao and Glazer 1996;). The remarkable decrease in the emission of the phycobiliproteins indicates the formation of degradation products from the high molecular mass aggregates which break down into hexamers $(\alpha\ \beta)_6$ which then disintegrate into trimers $(\alpha\ \beta)_3$ and eventually into monomers (Mörschel *et al.* 1980, Glazer 1989, Sinha *et al.* 1995b). This also indicates uncoupling of efficient energy transfer in individual phycobiliproteins within the phycobilisomes. In parallel, the decrease in the absorption and fluorescence peaks indicates the destruction of the chromophores. However, the first step in the breakdown of phycobilisomes is probably the destruction of linker proteins as has been reported earlier (Sinha *et al.* 1995 b,c, 1997). Many cyanobacteria can change the composition of their phycobiliproteins in response to light and nutrient limitations, and UV-B has also been reported to cause changes in phycobiliproteins (Nultsch and Agel 1986, Sinha *et al.* 1995b).

The irradiation of the artificial UV-B source applied in the present investigation may be higher than that of solar irradiation and not intended to simulate solàr radiation, but the applied irradiance in the present investigation could be comparable to one expected in the tropical rice growing countries particularly during hot summer seasons. Thus any substantial destruction of the stratospheric ozone layer might drastically affect the cyanobacteria in the rice paddy fields and this in turn will have adverse effects on the agricultural economy of developing countries where cyanobacteria are being considered as an alternate source of nitrogenous fertilizers for paddy fields and other crops (Sinha and Häder 1996). It would be worthwhile to study the long term impacts of UV-B irradiation on cyanobacteria under natural conditions.

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Sarcocystis spp. in Antelopes from Southern Africa

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Summary. Five *Sarcocystis* spp. recovered from muscle fibres of antelopes in southern Africa are described based on light and transmission electron microscopy. *Sarcocystis woodhousei* and *S. gazellae* in *Antidorcas marsupialis* from Namibia are described again and re-established as a recognizable species. A *Sarcocystis* sp. with hair-type cysts was found in *Antidorcas marsupialis* from Namibia and in *Aepyceros melampos* from South Africa. *Sarcocystis melampi* sp. n. is described in *Aepyceros melampos* from South Africa. This new species is characterized by a novel ultrastructural type of the sarcocyst wall which shows villar protrusions without any specific content, in the form of flat, wart-like elevations provided with projections folded over them. The possibility of the occurrence of *S. hominis* of cattle in African antelopes is discussed in connection with the finding of sarcocysts which are structurally indistinguishable from *S. hominis* in *Tragelaphus strepsiceros* in South Africa.

Key words: antelopes, *Sarcocystis gazellae, Sarcocystis hominis, Sarcocystis melampi* sp. n., *Sarcocystis* sp., *Sarcocystis woodhousei.*

INTRODUCTION

Antelopes have been known as intermediate hosts of *Sarcocystis* spp. for a long time, mainly in Africa. Out of 4 named *Sarcocystis* spp. found in Africa (Levine 1986, 1988; Odening in press) one turned out to be a *nomen nudum* (*"S. ruandae"*, see Chiwy and Colback 1926). The other 3 as well as the numerous unnamed forms are insufficiently characterized. Although there are a few descriptions by light microscopy worth mentioning for some of the forms (Benko 1968, Mandour and Keymer 1970, Kaliner 1975) it is difficult to recognize the species or forms because the diagnostically important ultrastructure of the sarcocyst wall (Dubey *et al.* 1989, Dubey and Odening in press) is unknown or only published as abstracts or short papers without any figures (Markus *et al.* 1984, 1985; Daly and Markus 1990). Three species (2 named and one unnamed) known in the Mongolian gazelle from Mongolia (Odening *et al.* 1996a) have been clearly identified. This article presents the results of light (LM) and transmission electron (TEM) microscopic examinations of sarcocysts found in the springbuck, the impala, and the greater kudu from southern Africa. The investigations were carrjed out during the course of a joint project between the Veterinary Investigation Centre in

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Skukuza, Kruger National Park, South Africa, and the Institute for Zoo and Wildlife Research in Berlin, Germany.

MATERIALS AND METHODS

The antelopes (Artiodactyla: Ruminantia: Bovidae; classification according to Wilson and Reeder 1993) were sampled during regular disease surveillance activities: 3 impalas (*Aepyceros melampus*, Aepycerotinae) and a greater kudu (*Tragelaphus strepsiceros*, Bovinae) in the Kruger National Park, South Africa, and one springbuck (*Antidorcas marsupialis*, Antilopinae) in Okandukaseibe (N.W. of Windhoek), Namibia. The tissues were fixed in 10 % buffered formalin and sent to Berlin. Several tissue samples and isolated sarcocysts were processed for histology. For transmission electron microscopy tissue samples and isolated sarcocysts were washed several times in phosphate buffer. Thereafter, they were post-fixed in 2 % osmium tetroxide, dehydrated with ethanol, and embedded in glycidether. Semi-thin sections were stained with toluidine blue. The ultra-thin sections were examined with a Zeiss EM 902 A.

RESULTS

Sarcocystis woodhousei Dogiel, 1915, emend. Wenyon, 1926 (Figs. 1-6)

Intermediate host: *Antidorcas marsupialis* (springbuck).
Site: intercostal musculature.
Locality and date: Namibia, July 1995.

Description: sarcocysts microscopic, 460-850 µm long and 60-100 µm wide, divided into compartments, 22-30 x 42-48 µm in size. The cyst wall, i. e. the ground substance plus the primary cyst wall (PCW), without the zone of the villar protrusions (P) was 0.3-0.9 µm wide. Tooth-like villar protrusions with irregularly shaped outlines arose at short distances from the cyst wall (Figs. 1, 2). They were 0.9-7.0 µm long and 1.3-4.5 µm wide and contained clusters of greater granules and osmiophilic, cord-like condensations, both variable in extent and shape (Figs. 3-6). The villar protrusions showed a hexagonal cross-section and their ensemble appeared like a honeycomb in the top view (Fig. 2). Bradyzoites fusiform or only slightly curved, 10-12 x 3.0-3.4 µm in size.

Sarcocystis gazellae Balfour, 1913 (Figs. 7-11)

Intermediate host: *Antidorcas marsupialis* (springbuck).
Site: intercostal musculature.
Locality and date: Namibia, July 1995.

Description: sarcocysts macroscopic, 1.4-2.8 mm long and 0.8-1.3 mm wide. The remnant of the host myofibre was surrounded by a layer of collagenous fibres with fibrocytes, representing a parasite-induced encapsulation of the host cell. The sarcocysts were divided into compartments (80-220 x 50-130 µm in size) by distinct septa (Figs. 7, 8). Within the ground substance (G) of the cyst wall small chambers (marginal chambers) were situated far of each other and at irregular distances. They contained only a few metrocytes (5-9 x 2.5-4.7 µm). The cyst wall (ground substance plus PCW, without villar protrusions) measured up to the beginning of the marginal chambers was 1-6 µm wide; including the marginal chamber zone it was up to 11 µm wide. The sarcocyst surface mainly appeared smooth (LM); by TEM finger-like villar protrusions without any specific content and with smooth surface arose occasionally from the cyst wall at irregular distances (Figs. 8-11). They were 1.9-4.8 µm long and 0.5-1.2 µm wide. The PCW between the villar protrusions had the usual small invaginations. The bradyzoites were only slightly curved and measured 12.2-16 (14.1±1.1) x 5-6 (4.5±0.7) µm in size (n = 15).

Sarcocystis sp. (Figs. 12, 13)

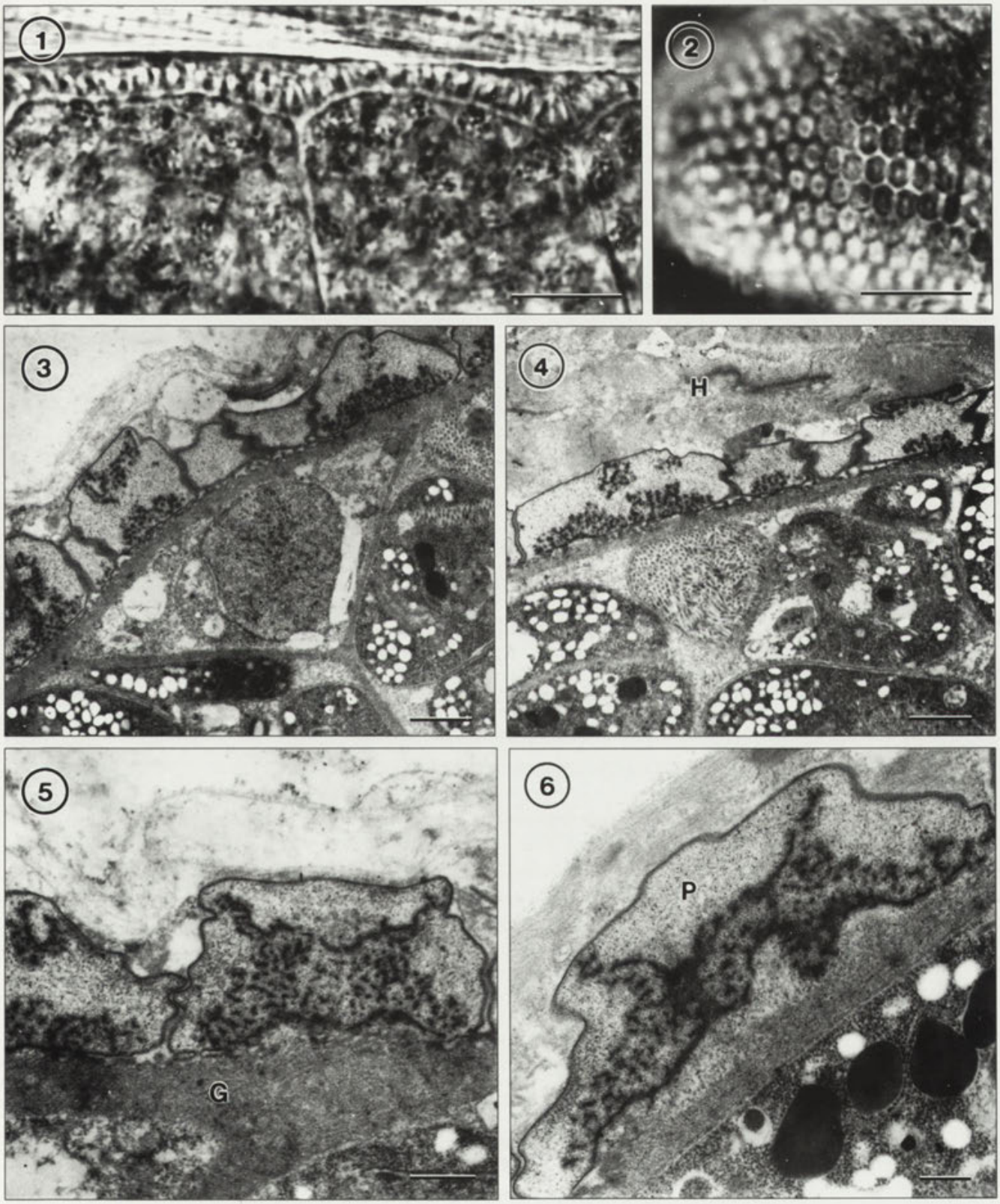
Intermediate host: *Antidorcas marsupialis* (springbuck), *Aepyceros melampus* (impala).
Site: intercostal musculature and diaphragm.
Locality and date: Namibia, July 1995; South Africa, July 1997.

Description: sarcocysts microscopic, 0.6-1.6 mm long and 75-110 µm wide. Compartmentation inconspicuous, compartments 20-35 x 30-50 µm in size. The cyst wall (ground substance plus PCW) was 0.3-1.0 µm wide. The sarcocyst surface was covered with hair-like villar protrusions without specific content and with a smooth surface (TEM type 6/7 according to Dubey *et al.* 1989 and Dubey and Odening in press). They were 10 µm long at least (LM) and 0.1-0.6 µm wide (TEM). The PCW between the villar protrusions showed the usual small invaginations. Bradyzoites slightly curved, 10.5-17.0 (12.8±1.9) x 3.2-4.5 (3.6±0.4) µm in size (n = 15).

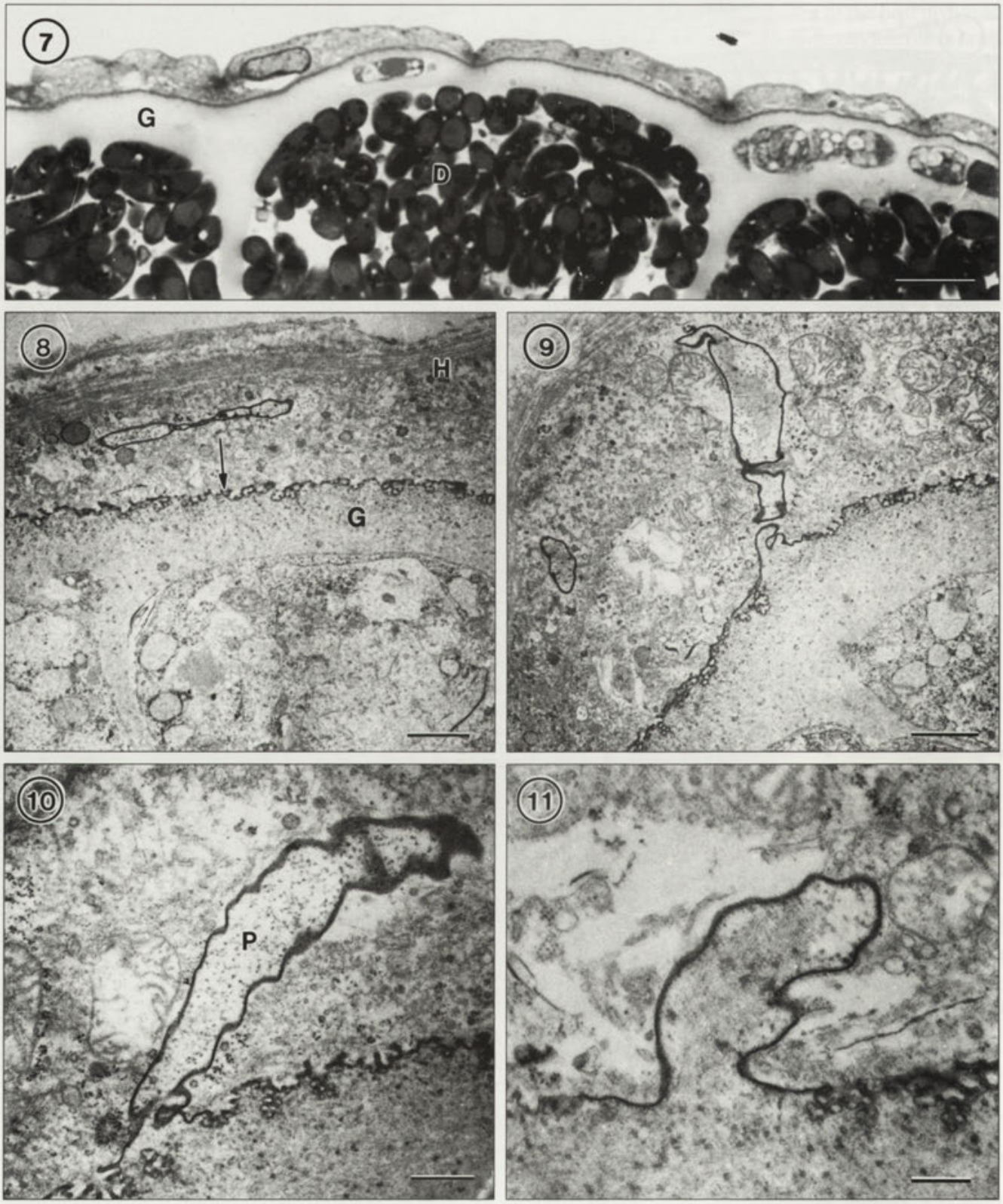
Sarcocystis melampi sp. n. (Figs. 14-18)

Intermediate type host: *Aepyceros melampus* (impala).
Site: oesophageal musculature.
Type locality and date: South Africa, Kruger National Park, July 1997.

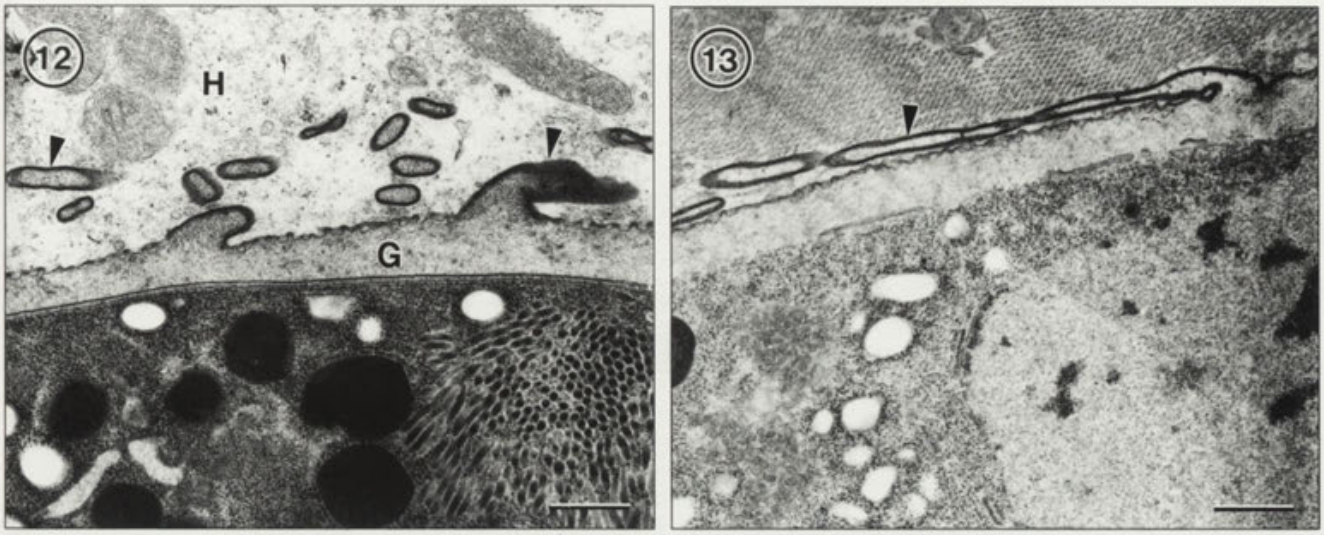
Description: sarcocysts microscopic, 0.6-1.6 mm long and 85-120 µm wide. The sarcocysts were distinctly divided into compartments (20-43 x 25-67 µm in size) (Fig. 14). The ground substance of the cyst wall including



Figs. 1-6. *Sarcocystis woodhousei* from *Antidorcas marsupialis*. 1 - photomicrograph of a part of an unstained intact sarcocyst, showing the cyst wall with slightly expressed villar protrusions. Scale bar - 10 μm. 2 -top view photomicrograph of a part of an unstained intact sarcocyst, showing the honeycomb-like arranged villar protrusions. Scale bar - 10 μm. 3-6 -TEM micrographs of the cyst wall region, G - ground substance, H - host cell remnant, P - villar protrusion. Scale bars - 3,4 - 1 μm; 5 - 0.5 μm; 6 - 0.3 μm



Figs. 7-11. *Sarcocystis gazellae* from *Antidorcas marsupialis*. 7 - photomicrograph of a semi-thin section of the cyst wall region. G - ground substance, D - compartment with bradyzoites. Scale bar - 10 μm. 8-11 - TEM micrographs of the cyst wall region. Arrow (8) points to the small invaginations on the cyst wall surface. G - ground substance, H - host cell remnant with connective tissue encapsulation on the top, P - villar protrusion. Scale bars - 8, 9 - 1 μm; 10 - 0.5 μm; 11 - 0.3 μm



Figs. 12, 13. *Sarcocystis* sp. from *Aepyceros melampus*. TEM micrographs of the cyst wall region with sections of the hair-like villar protrusions (arrowheads). G - ground substance, H - host cell remnant. Scale bars - 0.5 μm

the PCW was 0.4-1.7 μm wide. The sarcocyst surface was folded into villar protrusions with no specific content (Figs. 15-17). They appeared as flat, wart-like elevations which on their part were provided with projections folded over them (Figs. 16-18). The basic elevation of the folds, extensively seated on the cyst wall, was 4.6-7.0 μm wide. The layer of the villar protrusions was 1.0-2.5 μm high. The short, pit-like parts between the villar protrusions had the usual small invaginations. Bradyzoites slightly curved, 11.0-13.5 (12.6±0.8) x 3.2-3.4 (3.3±0.07) μm in size (n = 10).

Differential diagnosis: *Sarcocystis melampi* sp. n. is characterized by filiform microcysts with a new type of cyst wall ultrastructure compared to the classification by Dubey *et al.* (1989) and Dubey and Odening (in press). The new TEM-type is represented by villar protrusions without any specific content, in the form of flat, wart-like elevations with projections folded over them.

Specimens deposited: holotype as histological sections of a sarcocyst, Department of Pathology, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110, South Africa, no. WS1049/98. Paratypical material in the Institute for Zoo Biology and Wildlife Research, PF 601103, D-10252 Berlin, Germany.

Derivatio nominis: *melampi* is the genitive of the intermediate host species name *melampus*.

***Sarcocystis* cf. *hominis* (Railliet & Lucet, 1891) (Figs. 19-22)**

Intermediate host: *Tragelaphus strepsiceros* (greater kudu).

Site: diaphragm.

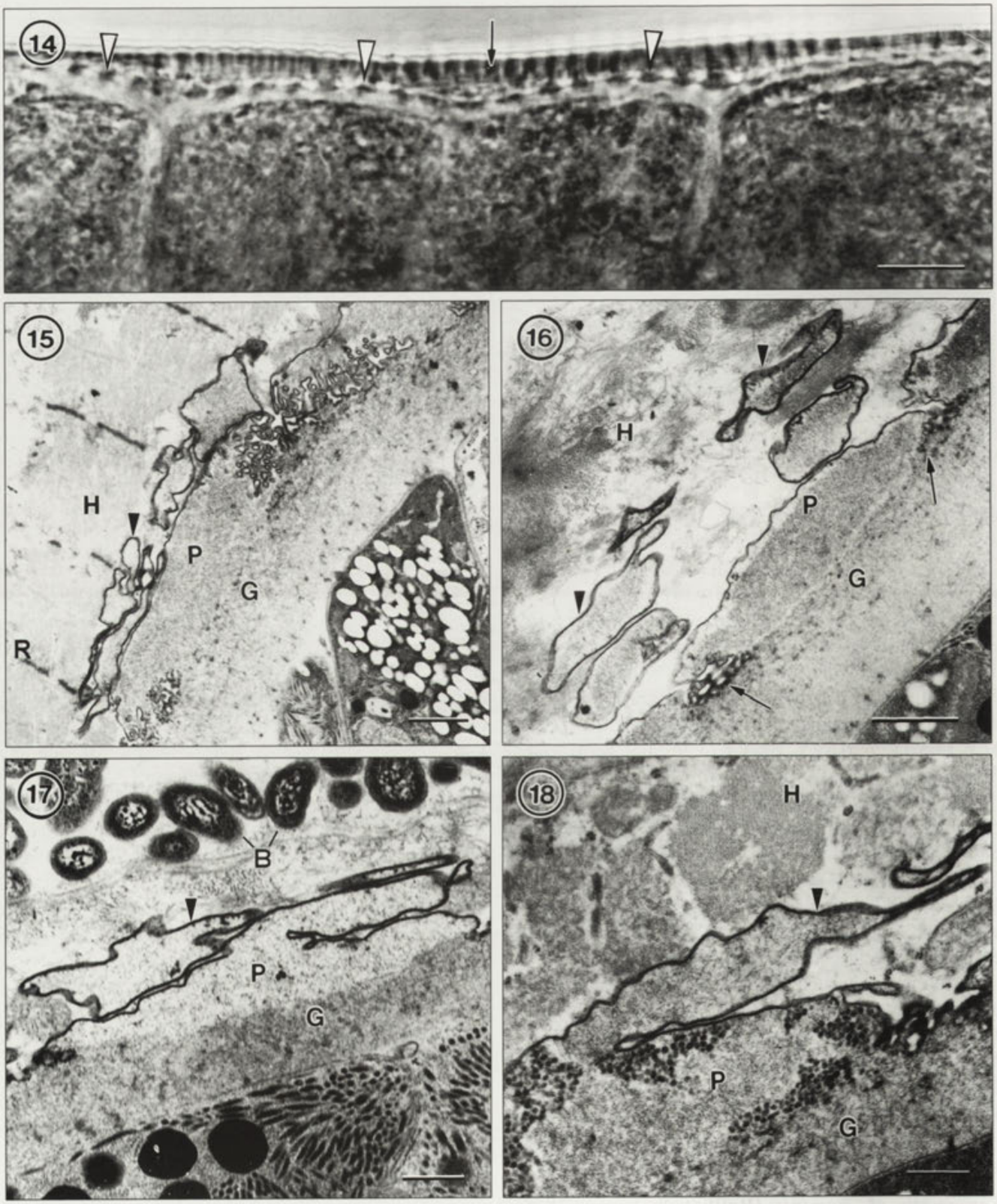
Locality and date: South Africa, July 1997.

Description: sarcocysts microscopic, 0.8-2.5 mm long and 90-145 μm wide. The sarcocysts were divided into compartments (10-33 x 25-36 μm in size). The ground substance of the cyst wall plus PCW was 0.9-2.3 μm wide. Villar protrusions, finger-like by LM and tombstone, club- or cone-like by TEM (Figs. 19, 20), arose from the cyst wall. They started with a broad base from the cyst wall, were tightly-packed and formed a palisade-like texture. They were 4.5-6.0 μm long and 1.3-4.8 μm wide and contained fine granules and microtubules in the interior (Fig. 21); their surface showed the usual small invaginations which were also found in the PCW between the villar protrusions (TEM type 10/15/16 according to Dubey *et al.* 1989 and Dubey and Odening in press). The bradyzoites (Fig. 22) were banana-shaped and 12-15 (14±0.9) x 2.6-3.0 (2.8±0.1) μm in size (n = 10).

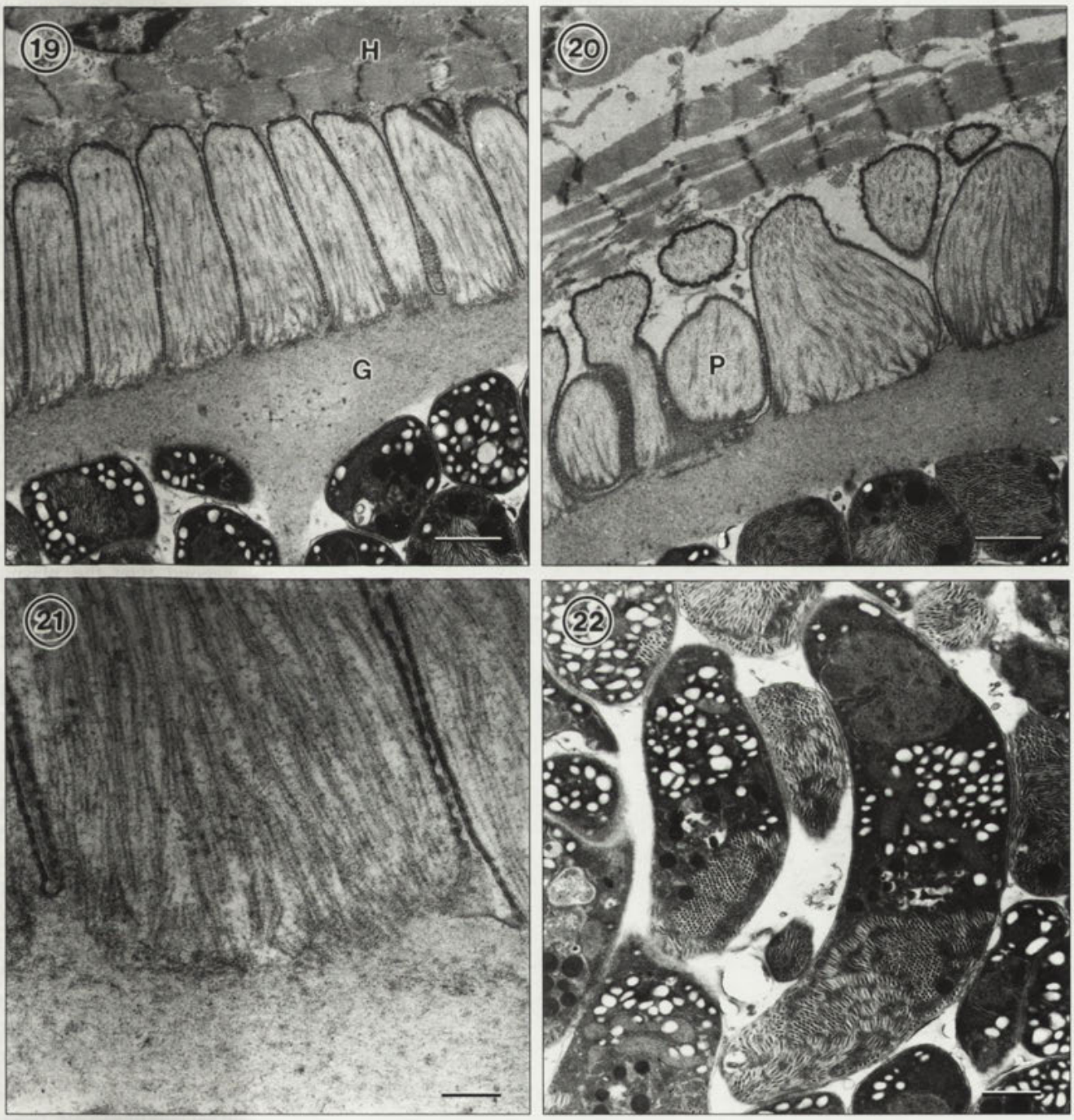
DISCUSSION

Following Levine (1986, 1988) who attributed several species of intermediate hosts belonging to the Reduncinae to *Sarcocystis nelsoni*, we re-constitute the previously scarcely recognizable species *S. woodhousei* and *S. gazellae* on the basis of closely related hosts and later descriptions.

Sarcocystis woodhousei. This species was described in 1915 as forming microcysts in the Grant's gazelle (*Gazella granti*) from East Africa. In spite of numerous subsequent



Figs. 14-18. *Sarcocystis melampi* sp. n. from *Aepyceros melampus*. 14 - photomicrograph of a part of an unstained intact sarcocyst, showing the cyst wall with wart-like villar protrusions (arrowheads). Arrow points to the remnant of the host myofibre. Scale bar - 10 μm. 15-18 - TEM micrographs of the cyst wall region. Arrowheads point to the projections of the villar protrusions folded over them. Arrows (16) point to the pits with small invaginations. B - bacteria, G - ground substance, H - host cell remnant, P - basal part of villar protrusion. Scale bars - 15, 16 - 1 μm; 17, 18 - 0.5 μm



Figs. 19-22. *Sarcocystis* cf. *hominis* from *Tragelaphus strepsiceros*. TEM micrographs of sarcocysts. 19 - palisade composed of narrow villar protrusions. G - ground substance, H - host myofibre. 20 - palisade composed of broad villar protrusions (P). 21 - basal part of a villar protrusion, showing microtubules in the interior and small invaginations on the surface. 22 - bradyzoites in the interior of a sarcocyst. Scale bars - 19, 20 - 1.6 μm; 21 - 0.3 μm; 22 - 1 μm

reports of sarcocysts in African antelopes this species has not been recorded again. It was only listed in a survey by Mandour and Keymer (1970) who mentioned that the radial striations of the sarcocyst wall (i. e., the villar protrusions) were expressed only slightly. Kaliner (1975) described microcysts in the Grant's and the Thomson's gazelles *(G. granti* and *G. thomsoni),* as well as in eland *(Taurotragus oryx),* bushbuck

(Tragelaphus scriptus) (="type 2 from Tragelaphinae"), and Kirk's dik-dik *(Madoqua kirki)* (="type 2 from Madoquinae") from East Africa. Their cyst walls appeared radially striated or homogenous and revealed honeycomb-like structures in tangential sections. A similar texture was described in *S. danzani* in the Mongolian gazelle (*Procapra gutturosa*) from Mongolia (Odening *et al.* 1996a, Stolte *et al.* 1996). The corresponding ultrastructure is classified as TEM type 29 according to Dubey and Odening (in press). The same ultrastructural type and the same honeycomb-like texture is present in our material from the springbuck which belongs to the same subfamily Antilopinae as the genera *Gazella, Madoqua,* and *Procapra*. Under the light microscope, the sarcocysts of all 4 cases (*S. woodhousei, Sarcocystis* sp. of Kaliner 1975, *S. danzani,* and our material) show a wall with more or less, but at most slightly expressed villar protrusions. We therefore consider the African forms as constituting the species *S. woodhousei* (intermediate hosts: *Gazella granti, G. thomsoni, Antidorcas marsupialis, Madoqua kirki,* and presumably also the Bovinae *Taurotragus oryx* and *Tragelaphus scriptus)*. The honeycomb-like texture and the ultrastructure of the cyst wall are characteristic for this species. This would also apply for *S. danzani* in Mongolia which is treated as a separate species due to the geographic distance. Nevertheless, the occurrence of similar sarcocyst forms in more or less closely related antelope species reflects the coevolution of *Sarcocystis* spp. with their intermediate hosts. Apart from sarcocysts in antelopes, TEM type 29 has been seen only in an unidentified species from the European hare (*Lepus europaeus*) (Odening *et al.* 1996b), and in *S. phacochoeri* from the warthog (*Phacochoerus aethiopicus*) in South Africa (Stolte *et al.* 1998).

Sarcocystis gazellae. This species was described in the red-fronted gazelle (*Gazella rufifrons)* in 1913 and has not been recorded afterwards again. It was only listed in a survey by Mandour and Keymer (1970), together with other findings of unnamed species from Antilopinae (*Gazella* spp.). The fat, ellipsoidal macrocysts from *Gazella* spp. listed by Mandour and Keymer (1970) probably represent the species *S. gazellae.* Apart from the original *S. gazellae,* these are *Sarcocystis* sp. of Benko (1968), *Sarcocystis* sp. of Mandour and Keymer (1970), and *Sarcocystis* sp. (thick-walled macrocysts) of Janitschke *et al.* (1976), all from *G. granti.* These forms are obviously identical with the macrocysts surrounded by a thin, homogenous wall which were found in *G. granti* and *G. thomsoni,* described by Kaliner (1975). Our samples from the springbuck correspond with these forms which all have a smooth surface under the light microscope in common. This is due to the sporadic arrangement of the villar protrusions. The individual villar protrusions correspond to those of the TEM types 4 or 14, according to the classification by Dubey *et al.* (1989) and Dubey and Odening (in press). Former data of the cyst wall suggest the presence of a connective-tissue encapsulation of the host muscle fibre which is verified in our material. A parasite-induced encapsulation of the host myofibre is known in most species with macrocysts (Odening *et al.* 1997; Stolte *et al.* 1997a, 1998; Bengis *et al.* 1998). This encapsulation, often already recognizable in young sarcocysts, is an important character for the determination of corresponding species, and two main types can be distinguished (Stolte *et al.* 1997a). In the first type, the plasma membrane of the host cell remains unaltered and a connective tissue layer follows peripherally. In the second type the plasma membrane of the host cell is distinctly thickened. *Sarcocystis gazellae* belongs to the former type.

Sarcocystis sp. The sarcocysts of this species represent the hair-type (LM) and the TEM-type 6/7, according to the classification by Dubey *et al.* (1989) and Dubey and Odening (in press). A similar species (with TEM-type 6/7 or 12) was mentioned by Markus *et al.* (1984). An identification is not possible on the basis of morphology alone. Sarcocysts of this type were described in *Procapra gutturosa* from Mongolia (Odening *et al.* 1996a) and in antelopes born in various German zoos (Stolte *et al.* 1997b). A corresponding form was mentioned in greater kudu *(Tragelaphus strepsiceros,* Bovinae) and gemsbuck *(Oryx gazella,* Hippotraginae) in southern Africa (Markus *et al.* 1984). The forms in free-ranging antelopes could belong to a separate species or possibly to *S. cruzi* from Bovini.

Sarcocystis melampi sp. n. Markus et al. (1984, 1985) mentioned the sarcocysts of two *Sarcocystis* spp. in impala from South Africa. At least one of them was transmitted to vultures as definitive host. One species had villar protrusions "flattened along the surface of the cyst", not unlike TEM-type 2/8 of the classification by Dubey *et al.* (1989) and Dubey and Odening (in press). The other species had mushroom-shaped villar protrusions (Daly and Markus 1990) and is similar to TEM-type 24 of the

above mentioned classification. Neither TEM-type 2/8 nor 24 is applicable to our material from impala. Kaliner (1975) found microcysts with very thin wall without any villi (LM) in the impala, Grant's and Thomson's gazelles in East Africa. These sarcocysts ("type 1 from Antilopinae") could belong to a species with the TEM-type 2/8 because the villar protrusions of this type are not visible by LM. The sarcocysts of "type 2 from Antilopinae" of Kaliner (1975) are distinctly different from those of *S. melampi* sp. n. and from those mentioned by Markus *et al.* (1984). A fourth sarcocyst form of Antilopinae described by Kaliner (1975) in the impala was not identifiable.

Sarcocystis cf. *hominis*. *S. hominis,* exhibiting TEM-type 10/15/16 according to the classification by Dubey *et al.* (1989) and Dubey and Odening (in press), is one of the 3 *Sarcocystis* spp. of cattle which occur globally. Contrary to some cases in which the intermediate host is only one species, the intermediate host specificity is broader in the 3 *Sarcocystis* spp. of cattle, extending to other species of the Bovini (Odening *et al.* 1995, 1998; Odening 1997, in press) as well. Thus, it seems likely that other Bovinae (apart from the Bovini) are also intermediate hosts of *S. hominis*. A sarcocyst form structurally indistinguishable from *S. hominis* was described in an eland (*Taurotragus oryx,* Bovinae) of unclear origin from a German zoo (Stolte *et al.* 1997b). This form was provisionally assigned to *S. hominis*. Markus *et al.* (1984) mentioned a sarcocyst in the greater kudu from Zimbabwe with features of *S. hominis*. Combined with our findings in the greater kudu from South Africa, this seems to be an indication that Bovinae other than Bovini are common usual hosts of a sarcocyst not discernible from *S. hominis* in Africa. These antelopes of the subfamily Bovinae are more likely hosts of *S. hominis* from cattle than hosts of a species from cervids (in Europe: *S. tarandi / S.hofmanni,* see Wesemeier and Sedlaczek 1995) which is morphologically not distinguishable from *S. hominis* (see Markus *et al.* 1984; Stolte *et al.* 1997b; Odening *et al.* 1998; Odening in press). No cervid species have evolved in Africa, whereas man's origin is situated in Africa. On the other hand, a further "sibling species" of *S. hominis* and *S. tarandi / S. hofmanni* could exist in African antelopes.

Acknowledgment. We thank National Parks Board of South Africa and their staff for their support in the field, and Marion Biering for her technical assistance. We are grateful to Dr. Manuela Stolte for the TEM micrographs of *Sarcocystis woodhousei* and *S. gazellae.*

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Diagnostic Characteristics of Owl Monkey (*Aotus trivirgatus*) Intestinal Trichomonads

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Summary. Intestinal trichomonads associated with disease in a colony of owl monkeys (*Aotus trivirgatus*) were investigated. Microscopic examination of fecal samples revealed that, whereas among normal monkeys their prevalence was low (2/30), it was high (38/44) among those with symptoms of gastroenteritis. Inasmuch as such studies have not been previously reported, several characteristics of organisms recovered in culture were compared with those of *Pentatrichomonas hominis*, the commonly found trichomonad in man and other host species. The owl monkey trichomonads differed from *P. hominis* in a number of aspects. For establishment in culture, they required residual live coliform bacteria or killed *Escherichia coli*. There was a wide variation in size and shape, and a pronounced tendency to form polymastigote units. The predominant number of anterior flagella was 4, although the counts ranged from 4 to more than 5. Attempts to demonstrate the independent flagellum by electron microscopy (EM) were unsuccessful. Only a small minority of *P. hominis* ingested human erythrocytes, whereas the owl monkey trichomonads were highly erythrophagocytic. The owl monkey trichmonads also exhibited cannibalism, as shown by EM. Because of these dissimilarities, the owl monkey trichomonad was named *Trichomonas aotus* sp.n.

Key words: intestinal trichomonads, owl monkey, *Pentatrichomonas hominis*, *Trichomonas aotus* sp.n.

INTRODUCTION

Intestinal trichomonads have a worldwide distribution in many host species including man, dogs, cats, rodents (Wenrich 1944), Old World monkeys (Dobell 1934), cattle (Jensen and Hammond 1964), swine, birds (Levine 1973), squirrel monkeys (Pindak *et al.* 1985), and the tree shrews (Brack *et al.* 1995). Detailed ultramicroscopic studies have revealed marked to only subtle differences, as well as similarities, between certain species; altogether, they have contributed to nomenclatural problems which arose about half-a-century ago. Some of them have not yet been fully resolved.

As evident from reviews by Honigberg (1978b, 1990), by far the most studied intestinal trichomonad has been the human parasite, *Pentatrichomonas hominis* Davaine, 1860. Before 1963, this protozoan has been described under numerous names, many of which have been judged to be synonyms of *P. hominis* (Honigberg 1963). *P. hominis* has been reported to occur in humans, various domestic and wild animals, among them Old World primates (Dobell 1934; Wenrich 1944, 1947; Kirby 1945; Flick 1954; Jensen and Hammond 1964; Honigberg *et al.* 1968; Wartoń and Honigberg 1979). Thus far, the squirrel

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monkey (*Saimiri* sp.) is the only New World primate in which intestinal trichomonads have been studied in depth; *Tritrichomonas mobilensis* has been recovered from nearly 100% of adult animals (Pindak *et al.* 1985, 1987; Culberson *et al.* 1986).

This report is concerned with intestinal trichomonads found in owl monkeys (*Aotus trivirgatus*), another species of New World primates. The trichomonads were obviously different from *T. mobilensis* and, in certain aspects, unlike *P. hominis*. Our data describe the features of these organisms and compare them with *P. hominis*.

MATERIALS AND METHODS

Test animals and sampling

Fecal samples from 30 healthy adult owl monkeys were collected immediately after their arrival from Battelle Pacific Northwest, a primate facility at Richland, Washington, USA, to the University of South Alabama Primate Research Center and unloading from the transport vehicle. Other 55 monkeys were sampled during an outbreak of gastroenteritis. The samples were collected with the aid of a sterile rectal swab and immersed in 3 ml of Medium 199 with 10% fetal bovine serum (FBS), 100 µg/ml kanamycin and 5 µg/ml amphotericin B.

After no more than 1 h maintenance at room temperature the specimens were vortexed briefly in order to disperse the fecal content and thus facilitate segregation of protozoa from the fecal matter which was allowed to re-settle during approximately 15 min standing. The top portion of the samples was then aspirated and split into 2 parts. One was used for direct microscopic examination. The other was for cultivation of the protozoa. It was inoculated into slant tubes (Nunc A/S, Roskilde, Denmark) containing a full monolayer of RK-13 cells and GMP medium; this method was used successfully for previous isolations of *T. mobilensis* from squirrel monkeys (Pindak *et al.* 1985). In addition, the second part of the samples from ill or dead monkeys was also used for detection of viruses. It was inoculated into tissue culture monolayers of commercially obtained HeLa, Hep-2, RD, human foreskin cells, and squirrel and owl monkey kidney cell cultures developed in our laboratory; the known combined susceptibility spectrum of these tissue cultures, demonstrable by the appearance of characteristic cytopathic effect, was wide enough to detect most of the commonly occurring human viruses, as well as of squirrel monkey adenovirus, cytomegalovirus, herpesvirus saimiri and herpesvirus tamarinus. Bacteriological studies of samples collected at autopsy were carried out in the Pathologist's diagnostic laboratory according to the guidelines from the Center for Disease Control, Atlanta, GA.

Microscopic examination

Wet amounts of the original specimens were examined by phase contrast microscopy (x 200 and x 400) for the presence of protozoa. Organisms from established cultures were studied by phase contrast and dark field (x 400), as well as by scanning (SEM) and transmission (TEM) electron microscopy.

Organisms studied

OM427 culture isolate from an owl monkey which died with symptoms of enterocolitis; it was used for detailed characterization. *P. hominis* ATCC no. 30000, used for comparisons with OM427. *Escherichia coli* ATCC no. 25922, serving as a culture medium supplement.

Cultivation of OM427 isolate

One ml portions of a pool from the third passage in RK-13/GMP medium were inoculated into 5 ml of media listed below. Flat side tubes (enabling direct microscopic examination of contents) were used for this purpose. The cultures were incubated at 37°C and examined daily. TYM, CTLM, Medium 199, GMP, MMP-4 and MMP-5 media were compared for the best support of growth. TYM and CTLM were prepared as formulated in the American Type Culture Collection Catalogue of Protists, 18th ed., 1993. Medium 199 was prepared from 10 x concentrate. The preparation of GMP medium has been described previously (Pindak *et al.* 1985). Composition of MMP-4 medium: Medium 199 (10 x concentrate) - 100 ml; tryptone concentrate (20 g/100 ml) - 100 ml; yeast extract concentrate (10 g/100 ml) - 100 ml; 200 mM L-glutamine - 10 ml; L-ascorbic acid (2 g/100 ml) - 2 ml; vitamin solution (Gibco BBL, Gaitersburg, MD)(100 x concentrate) - 10 ml; 50% glucose - 5 ml; D-pantothenate (2 g/100 ml) - 5 ml; L-cysteine HCl (10 g/20 ml) - 2 ml; lM HEPES buffer - 15 ml; sterile H_2O - q.s. 1000 ml. Tryptone and yeast extract were dissolved in distilled water and sterilized by autoclaving for 15 min. Other ingredients were filter-sterilized. The pH was adjusted to 6.8 with 5% NaOH. Except as specified otherwise, all materials were obtained from Sigma Chemical Co., St. Louis, MO. MMP-5 medium contained three parts of MMP-4 and one part of *Escherichia coli* culture grown in MMP-4, adjusted to pH 6.8, and killed during 24 h incubation in the presence of 100 µg/ml kanamycin. All media contained 10% heat-inactivated FBS.

Size determination

Live cultures were video-taped through phase contrast microscope at x 400 magnification. Length and width of individual organisms, as projected on video screen, were measured in mm. Prejudicial selection was avoided by measuring all organisms in a given screen field under motion-freeze mode. The screen magnification factor was calculated from a similar recording and measurement of a standard micrometer scale with 0.01 mm divisions. From the values so obtained it was determined that the magnification was x 1428, and therefore, 1 mm on the screen represented 0.7 µm in the actual size.

Detection of sialidase activity

A modification of Pereira's (1983) method was used as follows: 0,5 ml of filtered supernatant from 3 to 5-day-old cultures of OM427 trichomonads, in serial dilutions (1:1 to 1:16), were dispensed in 48-well flat bottom culture plates (Costar, Oxnard, CA); 0,1 ml of 2% human red blood cells (RBC) washed and resuspended in phosphate buffered saline (PBS) pH 7.0 was added to the test samples and to the control wells containing PBS alone, and PBS with 3 units of *Clostridium perfringens* sialidase (Sigma, St. Louis, MO). After 18 h incubation at room temperature, 4 agglutinating units of peanut lectin (Sigma, St. Louis, MO) were added to all wells. Following additional 2 h incubation, there was solid agglutination and adherence of RBC to the

well bottom in the sialidase-containing control; in PBS alone the RBC remained in suspension.

Detection of hemagglutinins

These tests were performed according to the procedure developed for demonstration of *T. nobilensis* hemagglutinin (Pindak *et al.* 1987). Dilutions of OM427 culture supernatant were prepared as described above for detection of sialidase. Phase contrast microscopy (x 400) of hemagglutination results from tests in which whole (uncentrifuged) cultures of OM427 trichomonads were used revealed an unexpected definite bonding between the organisms and human RBC type O. This phenomenon suggested further investigation, and comparisons with *P. hominis*.

Interactions with RBC

Three parameters were studied: (a) depletion of free RBC from a mixed suspension with trichomonads; (b) enumeration of RBC-positive trichomonads, defined as organisms with attached RBC, and of those free of RBC (RBC-negative); (c) phagocytosis of RBC by the trichomonads, demonstrated by light microscopy of 1 µm sections of embedded preparations stained with toluidine blue, and by TEM. Two-day-old cultures of OM427 isolate and *P. hominis*, both grown in MMP-5 medium, were harvested by centrifugation. Pelleted organisms were resuspended in PBS or in the spent culture medium and adjusted to 2 x 10^6/ml concentration. PBS-washed human RBC type O were adjusted to 2 x 10^7/ml concentration. Equal volumes of trichomonads and RBC were mixed and incubated for 1 h at 37°C. Respective counts were made in a hemocytometer chamber at x 400 magnification. The trichomonads were immobilized by a small amount of 1:10 diluted formalin.

Steps (a) and (b) were also performed with human RBC types, A, B, AB, and with rat, rabbit and dog RBC.

Sample preparation for electron microscopy

TEM: trichomonads were pelleted by centrifugation (800 x g, 10 min), washed three times in 12 ml PBS, and fixed in 3% glutaraldehyde in 0.2 M cacodylate buffer; post-fixed (1 h) in 1% osmium tetroxide; washed in cacodylate buffer; dehydrated in ethanol; embedded in Poly/Bed 812, and polymerized (18 h) at 60°C. Sections (60-80 nm) were cut on a Leica Ultracut microtome, stained with uranyl acetate and Reynolds lead citrate, and examined in CM 100 Phillips transmission microscope.

SEM: samples were harvested, washed and fixed as for TEM. One drop of the preparation was applied to coverslips coated with poly-L-lysine and kept for 18 h at 4°C, then washed in 0.1 M cacodylate buffer, post-fixed (30 min) in 1% osmium tetroxide and washed in cacodylate buffer. After dehydration in ethanol, they were dried in Denton Critical Point dryer, coated with gold-palladium in Denton DSM-5A cold sputter module, and examined in XL-20 Phillips microscope.

RESULTS

Microscopy of stool samples

Phase contrast microscopic examination of samples from 30 normal monkeys at their arrival revealed very few trichomonad-like organisms in 2 individuals. In contrast, during an outbreak of illness occurring approximately 2 months later, considerably higher numbers of organisms were seen in samples from 38/44 monkeys with symptoms of gastroenteritis, and in 2/11 asymptomatic control monkeys.

Cultivation and axenization

Primary cultures of trichomonads were established after 3-5 days of incubation. At that time all cultures contained enteric bacteria. Those with excessive overgrowth despite treatment with tobramycin were discarded. Subsequent treatment of less contaminated cultures with ceftazidime rescued 5 isolates which were further propagated with minimal contamination. Of those, isolate OM427 was selected for further studies. It was found to contain a single contaminant, identified as *Xanthomonas maltophilia*. The culture was axenized during 12 days of cultivation in the presence of 10 µg/ml ofloxacin. During that time, however, there appeared a progressive decline in the number of motile trichomonads. This phenomenon suggested dependence of the trichomonads on the presence of coliform bacteria. That assumption was supported by resumed proliferation after addition of 1 ml of *E. coli* culture previously killed with kanamycin.

The culture was transferred into Medium 199, GMP, TYM, CTLM, MMP-4, and MMP-5 media. While the trichomonads survived and apparently multiplied to some extent in all of them, only in the MMP-5 medium there was enough growth to carry the culture beyond the third passage. This medium was chosen for further routine cultivation of the organism.

Morphological features

Phase contrast microscopy (x 400) showed a wide variation in size and shape of the organisms. They ranged from small oblong individuals with undulating membrane and free axostyle to large round forms. Rapid outward pulsing of finger-like projections was clearly visible. The forward movement of the small organisms (up to 20 µm long) was relatively smooth. The motion of the large round forms (over 30 µm wide) was primarily rotary and tumbling. An axostyle was not evident in these individuals.

As viewed in dark field illumination, the small organisms showed clusters of predominantly four anterior flagella and a well developed undulating membrane terminating close to the tapered axostyle, then followed by a long recurrent flagellum. Occasional organisms with five anterior flagella were also present. Individuals of intermediate size (20-30 µm long) had two or more sets of flagella

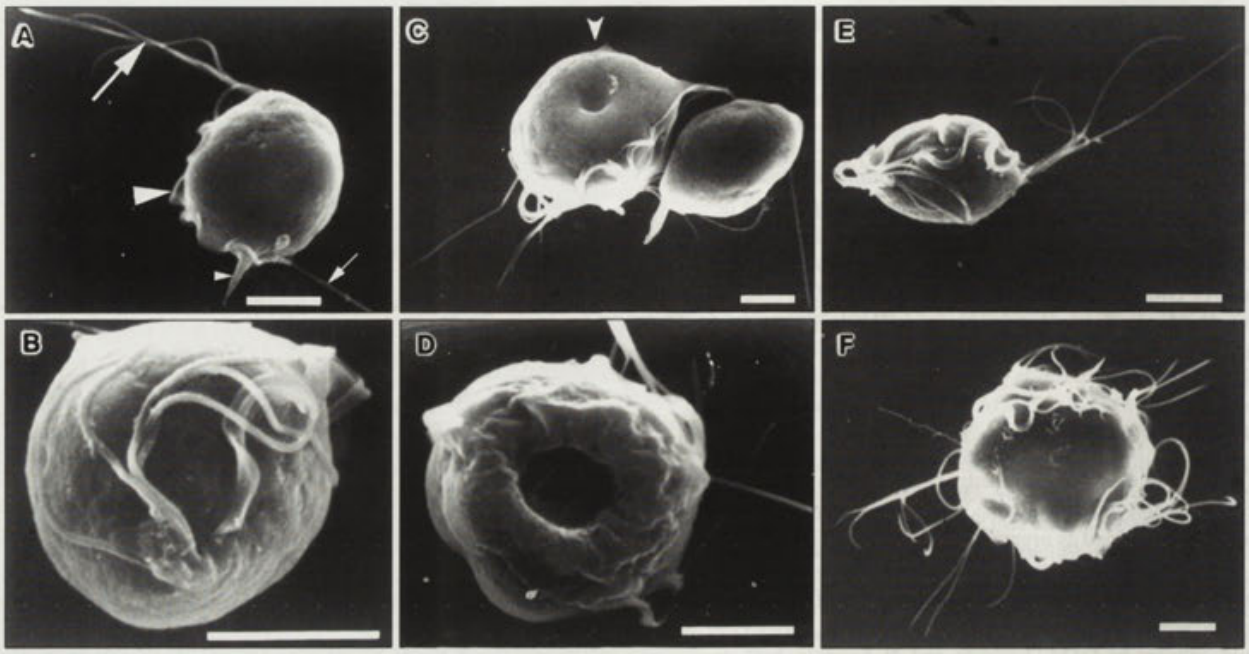

Fig. 1. Scanning electron microscopy of OM427 trichomonads. A - typical organism with 4 anterior flagella (big arrow), undulating membrane (big arrowhead), recurrent flagellum(small arrow) and axostyle (small arrowhead); B - an organism with 5 anterior flagella; C - opened alimentary crater (arrowhead); D - vertical view of alimentary crater; E - replicating organism showing 2 sets of flagella; F - a typical large organism with multiple sets of locomotor organelles. Scale bars - 4μm

and undulating membranes. A striking feature in the early culture passages were large round organisms, termed giant polymastigote bodies (GPmB), with numerous undulating membranes and clusters of four flagella. The largest GPmB seen measured 495 x 485 μm. When ruptured, the contents of the GPmB were quickly released, leaving only the collapsed cytoplasmic membrane.

SEM demonstrated the location and number of anterior flagella, the tendency to form polymastigote units, and a frequently seen conspicuous opening on the surface of the body, here named "alimentary crater" (Fig. 1). The appearance of the organism revealed by TEM is shown in Fig. 2.

Length and size distribution

Mean measurements of length and width and estimates of body size from calculated total visible area of OM427 and *P. hominis* are presented in Table 1. The entire

Table 1. Size distribution of live OM427 trichomonads and *P. hominis*

Organism	Length interval	Length x width mean (μm)	Mean surface area (μm²)*	Number (n)	Percent (±S.E.)**
OM427	≤15μm	13.1 x 8.8	362	127	55.0 (±3.3)
	≤20μm	17.0 x 11.2	598	63	27.3 (±2.9)
	≤25μm	21.7 x 16.0	1,091	21	9.1 (±1.9)
	≤30μm	28.1 x 24.1	2,127	10	4.3 (±1.3)
	>30μm	37.3 x 34.6	4,054	10	4.3 (±1.3)
				Σn = 231	
P. hominis	≤15μm	12.4 x 10.1	393	29	48.3 (±6.4)
	≤20μm	17.2 x 14.2	767	31	51.7 (±6.4)
				Σn = 60	

*Area = (π) (x̄ length) (x̄ width)

**Standard error = $\sqrt{\frac{\%(100-\%)}{\Sigma n}}$

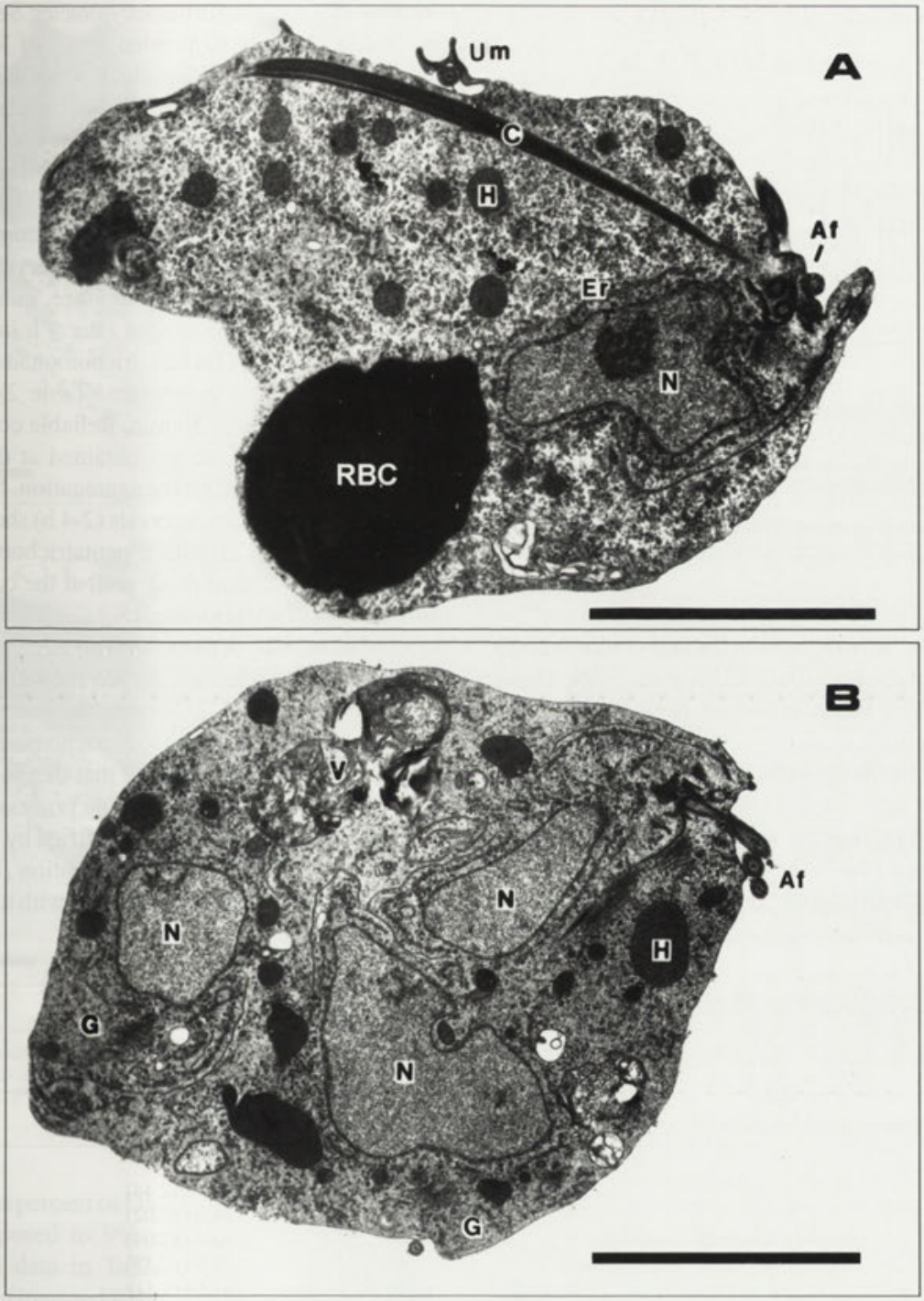

Fig. 2. Transmission electron microscopy of OM427 trichomonads. A -longitudinal section showing anterior flagella (Af), undulating membrane (Um), prominent costa (C), nucleus (N), endoplasmic reticulum (Er), hydrogenosomes (H), and ingested red blood cell (RBC); B - medium-sized organism with anterior flagella (Af), 3 nuclei (N) and Golgi complex (G), hydrogenosomes (H), and food vocuole (V). Scale bars - 4μm

measured population of *P. hominis* was rather uniform and fell into two numerically nearly equal groups, i.e., those shorter or longer than 15 μm. Although most of the OM427 trichomonads were within the range of *P. hominis,* 18% were considerably larger.

Interactions with erythrocytes

Previous studies of intestinal trichomonads commonly occurring in squirrel monkey have characterized two sialidase-producing strains of *T. mobilensis* differing in

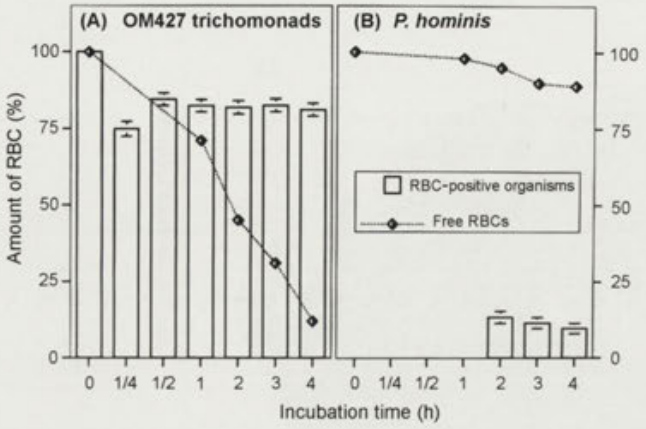

Fig. 3. Different interactions of OM427 trichomonads and *P. hominis* with red blood cells (RBC). A - the removal of free RBC by OM427 organisms was rapid and continuous while, in the presence of *P. hominis* (B), it remained at a minimal level. Similar difference was seen in the numbers of organisms with which RBC were associated

their release of a hemagglutinin (lectin) (Pindak *et al.* 1987, 1988; Demeš *et al.* 1989; Babál *et al.* 1994; Babál and Pindak 1995). When tested by the methods cited above, filter-sterilized culture supernatant of OM427 trichomonads contained neither a hemagglutinin nor a sialidase. On the other hand, firm cytadherence occurred between the trichomonads and RBC. Mixtures of RBC and trichomonads (10:1 ratio) were incubated at 37°C for up to 4 h. At times shown in Fig. 3, aliquots were withdrawn for counting in hemocytometer. Separate counts were made of freely suspended RBC, of RBC positive trichomonads, and of those which were RBC negative.

In 1 h, in the presence of OM427 trichomonads the number of free RBC was markedly decreased. Their depletion progressed steadily, to 12% of the original input at 4 h (Fig. 3A). In comparison, the count decrease of RBC incubated with *P. hominis* remained minimal throughout the experiment (Fig. 3B). The observed differences were not likely due to hemolysis since, in both samples, only few lysed RBC were seen after 4 h incubation.

The majority of OM427 trichomonads were RBC positive after 15 min incubation (Table 2). Their peak (84.6%) was reached at 30 min. Reliable counts of RBC positive *P. hominis* were not obtained at the first three intervals because of excessive aggregation. Nevertheless, the data for the last three intervals (2-4 h) showed that the proportions of RBC positive pentatrichomonads were significantly lower than those seen at the corresponding times in OM427 trichomonads.

Correlation of the depletion of free RBC and the counts of RBC positive trichomonads are shown in Fig. 3. The steady decline of free RBC during the interval where the numbers of RBC positive OM427 trichomonads remained essentially unchanged indicated that they were removed by the trichomonads in a continuing process of phagocytosis. The ingestion of RBC was verified by light microscopy of samples fixed after 2 h incubation , embedded in resin, cut in 1 μm sections, and stained with toluidine blue.

Table 2. Time-related uptake of RBC by OM427 trichomonads and *P. hominis*

Organism	Incubation time	Σn*	RBC-positive organisms		
			n	%(±S.E.)**	P-value
OM427	15 min	315	236	74.9 (±2.44)	<0.005[a]
	30 min	318	269	84.6 (±2.02)	
	1 h	347	286	82.4 (±2.04)	NS
	2 h	320	262	81.9 (±2.15)	NS
	3 h	322	266	82.6 (±2.11)	NS
	4 h	345	280	81.2 (±2.10)	NS
P. hominis	2 h	293	39	13.3 (±1.98)	<0.001[b]
	3 h	330	38	11.5 (±1.76)	<0.001
	4 h	319	31	9.7 (±1.66)	<0.001

* Σn - sum of all organisms; n - number of RBC-positive organisms
** Standard error calculated as in Table 1
[a] Differences from 30 min within OM427, determined by 2 x 2 Chi square test
[b] *P. hominis* differences from OM427 at corresponding time intervals
NS - not significant

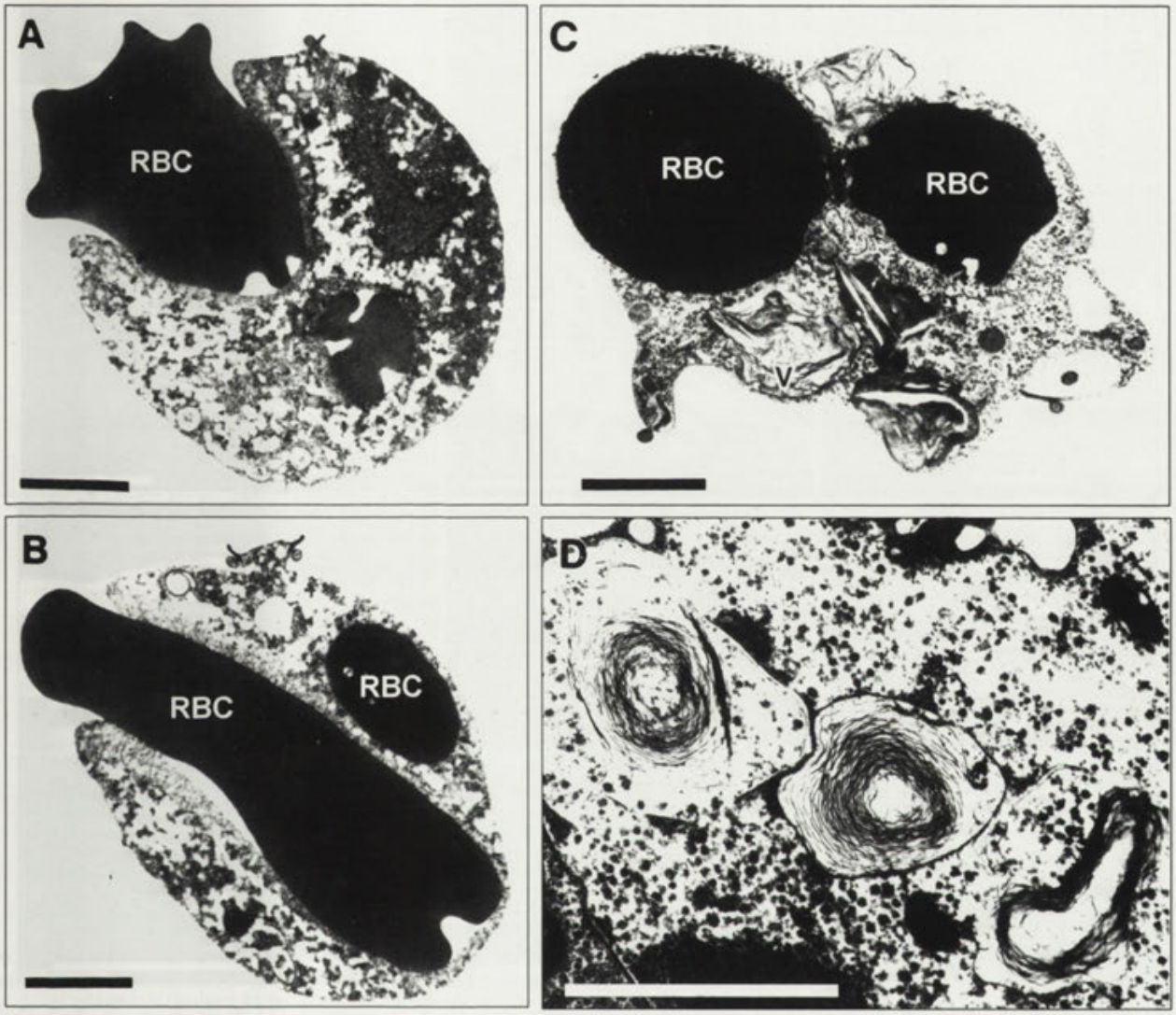

Fig. 4. Transmission electron microscopy of erythrophagocytosis. A - initial engulfment of red blood cell (RBC); B - deep insertion of RBC within the organism; C - completely internalized RBC and food vacuoles (V) filled with RBC remnants; D - high magnification of myelin-like remnants of ingested RBC ("myelinoid membranes"; see Ghadially 1988). Scale bar - 2μm

Seventy-three percent of OM427 trichomonads contained RBC, as opposed to 9% of *P. hominis*. These values reflected the data in Table 2. TEM demonstrated the localization of ingested RBC within the trichomonads and their disposal (Fig. 4): RBC were acquired through the opened alimentary crater (A, B); after being fully internalized (C) and digested, their remnants were seen as concentric strands of a myelin-like substance within well-defined vacuoles (D); these resembled closely illustrations and description of osmiophilic laminated "myelinoid membranes" representing breakdown products of phagocytosed erythrocytes (Ghadially 1988 [pp. 589-765]).

The sequence of events involved in the process of RBC ingestion was reconstructed from observations made by SEM (Fig. 5). Briefly, individual RBC were swept by the anterior flagella to the body surface where they became bound by a hitherto undescribed receptor (A, B). An outward pulse of a cytoplasmic thrust occurring near the attached RBC retracted, leaving a temporary alimentary crater. The reversed flow of cytoplasm in the crater pulled the attached RBC inwards (C, D). The crater closed when the RBC was fully internalized (E, F). This process was repeated until, presumably, the holding capacity of the organism was reached. These conclusions were substan-

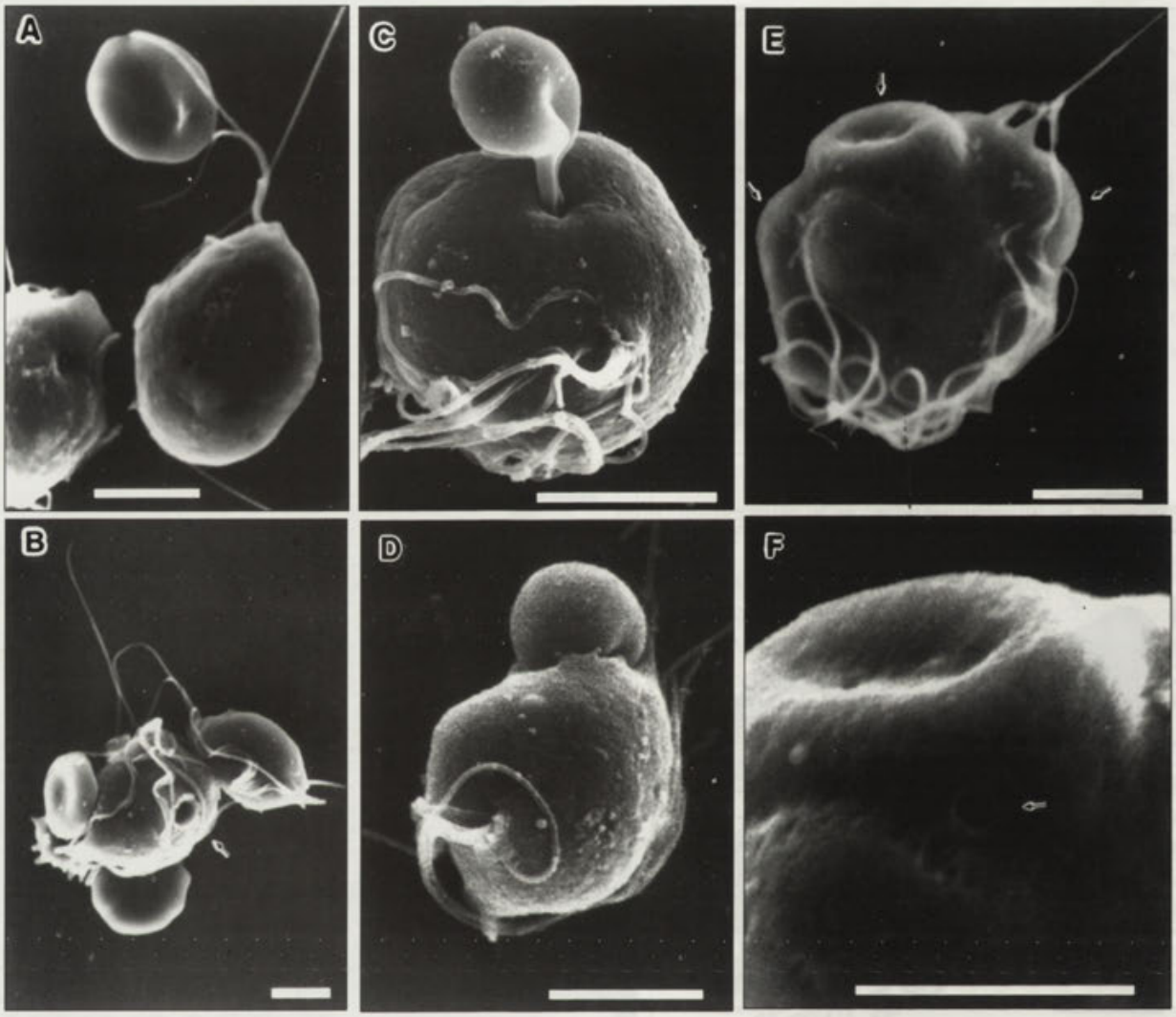

Fig. 5. Scanning electron microscopy sequence of erythrophagocytosis. A - contact of red blood cell (RBC) with anterior flagella. B - deposition of RBC on the body surface, arrow indicates an open alimentary crater; C - initial stage of RBC internalization: distorted RBC "pulled" into alimentary crater (see also Fig. 4 A, B); D - late stage of RBC internalization, note irregular rim of alimentary crater; E - organism silhouetting several fully internalized RBC (arrow); F - detailed view from E showing nearly closed alimentary crater (arrow). Scale bars - 4μm

tiated by relevant observations *in vivo*. The sweeping motion of extended flagella toward the distant part of the body was clearly seen in dark field illumination. Individual RBC, when within the reach of the flagella, were moved to the body of the organism where they remained attached. After this process was repeated a number of times, it was not unusual to see an organism nearly covered with RBC.

In addition to erythrophagocytosis, the larger polymastigote forms were also capable to cannibalize entire smaller organisms, as shown in Fig. 6. It presents a large organism with identifiable undulating membrane and a digestive vacuole. Entirely withing its confines is another organism; the essential feature is the ingested RBC which it contains: that RBC could have not been phagocytosed, unless the organism first was on the outside where it had access to it. Subsequent ingestion, i.e., cannibalism of the RBC-containing organism by the larger one explains its depicted location.

The results of comparative test for bonding of OM427 trichomonads with other than type O human RBC (number RBC positive/total number) were as follows: control (type O) - 123/171 (71.9%); type A - 112/159 (70.4%); type B - 122/168 (72.6%); type AB - 116/164

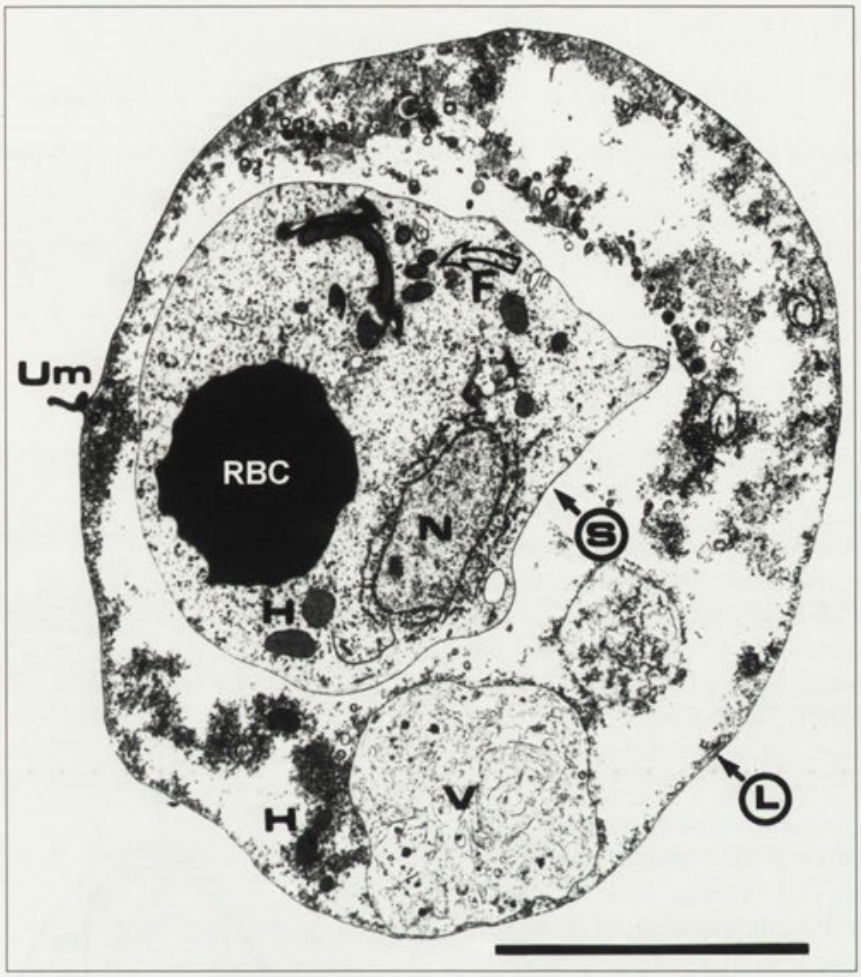

Fig. 6. Transmission electron micrograph of cannibalistic OM427 trichomonad. Large organism (circled L) with undulating membrane (Um), food vacuole (V), hydrogenosomes (H), and cytoplasmic remnants. Cannibalized small organism (circled S) showing internalized flagella (F), nucleus (N) and endoplasmic reticulum, hydrogenosomes (H), and phagocytosed red blood cell (RBC). Scale bar - 10μm

(70.7%); dog - 101/146 (69.2%); rat - 107/168 (63.7%); rabbit - 96/153 (62.7%). (Owl monkey RBC were not available.) In all instances, the Chi square values obtained from comparisons with the control were less than 3.84, thus showing no significant difference from human type O RBC. The numbers of RBC positive organisms were significantly reduced, however, when human RBC were exposed for 6 h at 37°C to sialidase derived from *Clostridium perfringens*, or when the trichomonads were preincubated for 20 min with N-acetylneuraminic acid prior to addition of normal RBC (Table 3).

Summary of descriptive features

Approximately 80% of organisms grown in MMP-5 medium are pyriform (Fig. 7), with mean body length of 15 μm. The typical length-to-width ratio is 1.6 : 1. Four (rarely 5) anterior flagella (Af) are nearly as long as the body. The well developed undulating membrane (Um) terminates close to the "exit" of axostyle (Ax), and is followed by recurrent flagellum (Rf). Hydrogenosomes (H) are present in the vicinity of prominent costa (C) and axostyle. Pelta (P) is moderately broad. Golgi complex (G) is near the ovoid nucleus (N) surrounded by endoplasmic reticulum (Er). About 20% of organisms are round bodies of varied diameter, with more than one nucleus. A prominent feature of the organism is its ability to phagocytose and digest intact erythrocytes.

Host: *Aotus trivirgatus* (owl monkey), normally living in the Amazon Basin. All animals were adults reared in captivity. Less than 6 months prior to their transport to the

Table 3. Sialic acid-dependency of RBC uptake

Sialic acid concentration	OM427 trichomonads			*P. hominis*		
	RBC positive/total	% positive	% inhibition*	RBC positive/total	% positive	% inhibition
None	225/324	69.4	0	43/338	12.7	0
80 mM	69/431	20.2	70.9	38/314	12.1	4.7
40 mM	105/334	31.4	54.8			
20 mM	130/325	40.0	42.4			

* % inhibition = [1-(test % positive) ÷ (control % positive)] x 100

University of South Alabama Primate Research Laboratory, Mobile, Alabama, they were maintained at Battelle Northwest, in Richland, Washington.

Type culture: *Trichomonas aotus* stock OM427 has been deposited in the American Type Culture Collection, Rockville, Maryland, ATCC 50649.

Relation to disease

Although at the outset detailed epidemiological studies were not carried out, certain observations of relationship between the above described trichomonads and disease of the animals were made. Routine sampling of 30 serially chosen monkeys at the time of their arrival identified 2 individuals with very few intestinal trichomonads. Within a month, one monkey (not of the 30 previously examined) died with symptoms of gastrointestinal abnormalities. Samples from other animals with similar symptoms were examined during the outbreak. In total, high numbers of trichomonads were detected in 38/44 monkeys. The organisms were also found in 2/11 asymptomatic (control) animals. As determined by 2 x 2 Chi square test, the difference between the symptomatic and asymptomatic positive monkeys was significant ($P<0.001$). Twenty of the 85 examined monkeys died. (Among them was the individual from which isolate OM427 was recovered). Thirteen were found trichomonad-positive and 7 negative at previous times of examination.

The synopsis of findings obtained from necropsy of the monkey from which organism OM427 was isolated was as follows. The glandular portion of the stomach was thickened and showed extensive hemorrhage. The small intestine, colon and cecum contained dark mucoid fluid. There was no formed feces. The mesenteric lymph nodes in the ileocecal area appeared prominent and dark grey.

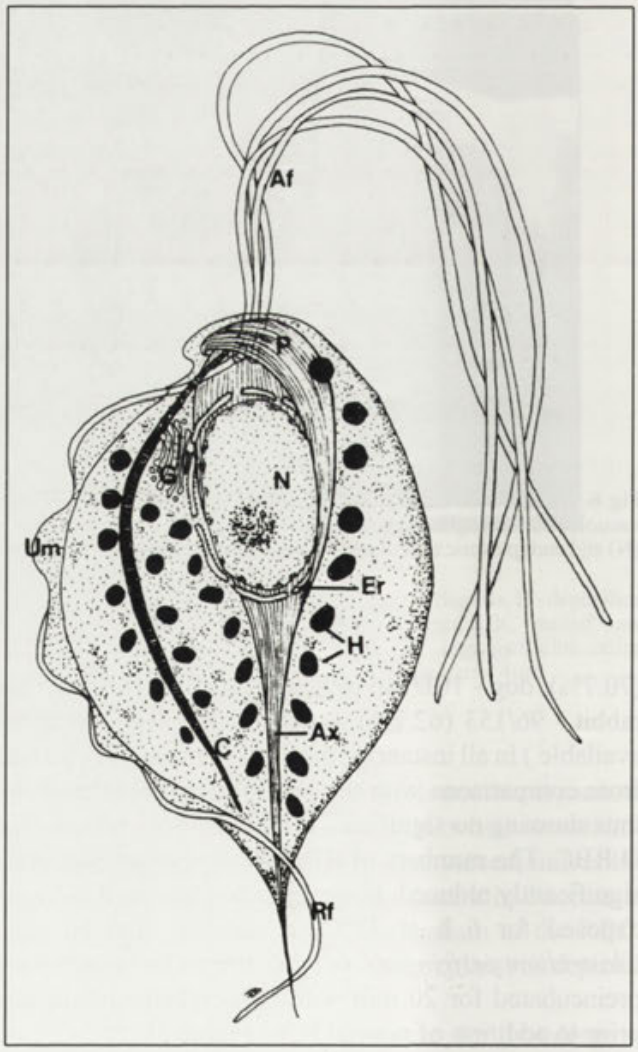

Fig 7. *Trichomonas aotus* sp. n. (OM427). Af - anterior flagella, Ax - axostyl, C - prominent costa, Er - endoplasmic reticulum, G - Golgi complex, H - hydrogenosomes, N - nucleus, P - pelta, Rf - recurrent flagellum, Um - undulating membrane

In the heart, the entire surface of the left ventricle had yellow/tan discoloration. The Pathologist's gross diagnosis was "gastroenteritis and myocarditis". Bacteriological and virological studies performed on fecal sample collected at necropsy did not identify any microbial agents to which the clinical findings could be attributed. Direct microscopic examination of the sample, however, revealed numerous trichomonads.

DISCUSSION

As far as we were able to determine, there are no previous reports describing intestinal trichomonads occurring in owl monkeys. The here reported high incidence of trichomonads during an outbreak of gastroenteritis in these New World primates prompted identification of the recovered organisms. Particular consideration was given to the possible presence of *P. hominis* because of numerous reports of its incidence in hosts of widely unrelated species (see Honigberg 1963, 1990 for extensive references), and to *T. mobilensis* (Culberson *et al.* 1986), originally recovered form squirrel monkeys (Pindak *et al.* 1985). In their natural habitat, owl and squirrel monkeys at times inhabit the same territory in the Amazon Basin. Fecal shedding of *T. mobilensis* by squirrel monkeys has been reported as very common (Pindak *et al.* 1985, 1987); since owl monkeys have the opportunity to ingest fecal matter of squirrel monkeys, it was considered that they thus may acquire *T. mobilensis*. Our results showed, however, that the owl monkey trichomonad was not related to *T. mobilensis*.

The counts of anterior flagella were determined by dark field microscopy of living cultures. While this method was deemed most reliable, the movement of the organisms in the visual field precluded exact categorization of the trichomonads with respect to the total numbers of the flagella in a manner similar to Wenrich's (1944) report. Nevertheless, it was clear that a high proportion had 4 anterior flagella. This and other characteristic argued against the consideration that the studied organism should be identified as *P. hominis*, despite the observation of 5 flagella in a minority of the culture population.

Seemingly ever since its discovery, *P. hominis* has had the distinction of being the most controversial among trichomonads; a brief review of its history seems appropriate for a better focus on the identification process of the owl monkey trichomonad. Its present name is credited to Wenrich (1931, 1944). In Wenrich's time, various erroneous claims still were made, such as, "Cultural forms of *T. vaginalis* are morphologically indistinguishable from *T. hominis*" (Andrews 1929, and others). On the other hand, Dobell (1934) expressed concern about the name of the human intestinal trichomonad (*P. hominis*) being readily applied to organisms recovered from non-human hosts by writing, "Unfortunately, it is now becoming fashionable to call all the intestinal protozoa of 'monkey' by the names of the corresponding forms found in men merely because they are not obviously different in structure" [p. 571].

The presently accepted fundamental knowledge about *P. hominis* can be traced primarily to Wenrich, Kirby, and Honigberg. It may be significant that all of their conclusions have been based strictly on morphological observations of trichomonads from human sources; Dobell's concern about pentatrichomonads from non-human hosts (quoted above) may still be valid. Wenrich (1931, 1944) introduced the notion that the "majority" of *P. hominis* have 5 anterior flagella in a "4+1" arrangement. Interestingly, this claim may be questioned when Wenrich's (1944) data are carefully examined: the "majority" (67.3%) seems to have been derived from a pool of data from three unrelated samples; two were "clones", each from a different individual; in the third one, a stool sample from yet another person, the organisms with 5 flagella represented 42% while 51% of the total had 4 flagella. Subsequently, Wenrich (1947) showed concern about the trichomonads with 4 and 3 flagella as possibly being different from *Pentatrichomonas* and wrote, "Until this is determined, I have chosen to use the name *T. hominis* for the intestinal group"[p. 180]. Kirby (1945) pointed out that, while 4 of the flagella are almost always grouped together, the 5th (independent) flagellum has a separate origin and is directed posteriorly, i.e., in the opposite direction from the other 4. As he stated, "This feature justifies generic distinction of this flagellate of man, as well as of similar forms in other animals" [p. 170]; at the same time Kirby was uncertain about the classification of organisms recovered from kangaroos and other non-human hosts because, although generally fitting the description of *P. hominis*, they showed subtle differences. Honigberg *et al.* (1968) demonstrated by TEM the location of the cluster of kinetosomes belonging to the 4 anteriorly oriented flagella, and the separate origin and course of the independent flagellum. These details were also shown by SEM (Wartoń and Honigberg 1979). Honigberg's (1963) reclassification of at least 30 names as certain or probable synonyms of *P. hominis* greatly alleviated the previously existing nomenclatural confusion.

In view of the rather confounding problems briefly mentioned above, identification of the OM427 owl monkey trichomonad required several studies in which the properties of this organism were compared with those of *P. hominis* ATCC no. 30000, the only strain of human origin listed in the American Type Culture Collection of Protists (18th ed. 1993). The undulating membrane of the OM427 trichomonad, like that of *P. hominis*, extended the full length of the body, and was followed by a free posterior flagellum. The two organisms differed, however, in the number of anterior flagella. Extensive efforts to demonstrate the independent flagellum by TEM (Honigberg *et al.* 1968) were unsuccessful. While the movement of *P. hominis* is commonly characterized as "jerky", that of OM427 trichomonads was relatively smooth; this feature may indicate absence of the independent flagellum which, being oriented posterolaterally, would be expected to exert thrust in a direction of its own, and thus interfere with the forward drive by the other 4 flagella of *P. hominis*. The external surface of *P. hominis*, as seen by SEM, has been shown to be smooth (Wartoń and Honigberg 1979). No previous mention has been made of the funnel-shaped alimentary craters commonly seen in the OM427 trichomonads (Fig. 1).

It is not clear to what extent the GPmB of OM427 trichomonads are related to somatellae which have been mentioned to occur in cultures of various trichomonads (Cleveland 1928, Stabler 1941, Wenrich 1944, Wirtschafter 1954, John and Squires 1978). No specific function has been ascribed to somatellae which are believed to reflect unfavorable environmental conditions suppressing cytokinesis (Honigberg 1963). The GPmB were frequently seen in early culture passages before complete axenization was ascertained. Their formation may be related to metabolic products of the initial contaminants, perhaps the frequently encountered *Pseudomonas* sp.; *Pseudomonas aeruginosa* lectins have been reported to stimulate growth of some protozoa (Gilboa-Garber and Sharabi 1980) and exert mitogenic effect (Sharabi and Gilboa-Garber 1979). The GPmB may also play a protective role in the survival of OM427 trichomonads. Phase contrast microscopy suggested that some contained small organisms trapped inside. Such cannibalistic behavior was also observed by videomicroscopy, and confirmed by TEM (Fig. 6). We are unaware of a similar previous documentation of cannibalism by *P. hominis*.

Diamond (1957) has reported TYM medium alone to be well suited for primary isolation of *P. hominis*. Fresh isolates of the owl monkey trichomonads propagated in this medium only when bacteria were present, either as live residual contaminants or killed *E. coli*. At present, MMP-5 still appears to be the medium of choice for routine propagation of the axenic OM427 culture. It is not certain that all of its components are essential. Limited experience has shown that the organism can be grown in TYM medium supplemented with killed *E. coli*. It remains to be determined whether the original properties of the trichomonads will be retained.

Substantial difference between *P. hominis* and the OM427 trichomonad was also evident from the comparison of their respective rates of RBC clearance and attachment to the organism (Fig. 3). Over the span of 4 h, most of added RBC remained free in the suspended mixture with *P. hominis*, whereas they were mostly removed when mixed with the owl monkey isolate. The RBC clearance was related to the proportions of RBC-positive organisms (Table 2). Specific details underlying the process of RBC attachment to the trichomonads will require further specific studies. Exploratory data (Table 3) indicated that only the OM427 trichomonads have a lectin-like sialic acid-specific moiety on their surface while the pentatrichomonads do not. At present, it is not known whether the flagella drive RBC to the body surface (Fig. 5 A, B) by simple mechanical force or also contain a specific RBC receptor. Detailed analysis of surface receptors of both organisms may provide another evidence of unrelatedness between the owl monkey trichomonad and *P. hominis*.

Phagocytosis of RBC by *P. hominis* has been mentioned in brief statements by Dobell (1934), Wenrich (1944), Caruso (1960) and others. None of these reports (or any that we were able to trace) contained data on which such conclusions have been made. As we have experienced, the most reliable method for distinction between ingested RBC and those which may be merely attached to the outside of a given organism is their demonstration within the confines of trichomonads by TEM or light microscopy of stained thin sections. Both methods were used successfully in this study. The key steps involved in erythrophagocytosis by the OM427 trichomonads (Figs. 4, 5) may be the first definitive proof of RBC ingestion by trichomonads.

The higher prevalence of the trichomonads in overtly diseased monkeys than in those which were asymptomatic was statistically significant. The animals were an essential part of a study which did not allow additional experiments necessary to test the causal relatedness of the trichomonad to the pathological findings.

The current taxonomy of intestinal trichomonads is based solely on morphological details. The diagnostic

criteria for *P. hominis* found in humans are well defined. As these developed, uncertainties began to emerge about applying the name *P. hominis* to some trichomonads from nonhuman hosts. In reference to the 5-flagellated trichomonads from kangaroos, poultry and termites, all bearing features of the human *P. hominis*, Kirby (1945) suggested that "Until comparisons are made with regard to those, as well as other features, it is not possible to state that all these forms are morphologically identical with one another" [p. 170]. Similarly, Honigberg (1963) stated that " .... one may question the validity of many species placed along with the human intestinal trichomonad in the genus *Pentatrichomas*" [p. 47]. In a later publication, Honigberg (1978a) also mentioned that placing the bovine intestinal trichomonads (Jensen and Hammond 1964) with *P. hominis* did not seem justified and predicted that "Physiologic and immunologic investigations may ultimately result in changes of the criteria employed for differentiation of trichomonad species, and this may lead to separation into species of structurally identical organisms within *Pentatrichomonas* and other genera of Trichomonadida" [p. 254-255]. In principle, the structural characteristics of the owl monkey trichomonad (summarized in Fig. 7) resembled those of *Trichomonas gallinae* (Mattern *et al.* 1967) which, except for the number of anterior flagella, are also to be found in *P. hominis* and other members of the subfamily Trichomonadinae (Honigberg 1978a [p. 169], 1978b [p. 409]). Therefore, ultrastructure alone was not adequate for identification of the organism here studied. The overriding distinction between *P. hominis* and the OM427 trichomonad was found in the comparative studies of their biological properties. Because of the substantial dissimilarities, the owl monkey isolate was named *Trichomonas aotus* sp.n.

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ACTA
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Trichodina mystusi, a New Species of Trichodinid Ciliophoran from Indian Estuarine Fish, *Mystus gulio* (Hamilton)

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Summary. *Trichodina mystusi* sp. n. is described from the gill-filaments of *Mystus gulio* (Hamilton) from Matla River, West Bengal, India. This trichodinid is disc- to bell-shaped and flattened in lateral view. The mean diameter of the body is 30.9 µm (27.6-36.8) consisting of 20-24 denticles. The shape of denticle and the appearance of central area is unique. There are 5-7 radial pins per denticle. The adoral spiral is 390-400°.

Key words: estuarine fish, India, *Mystus gulio*, parasite of fish, *Trichodina mystusi* sp. n., trichodinid.

INTRODUCTION

In publications of Hagargi and Amoji (1979), Mukherjee and Haldar (1982), Das and Haldar (1987), Das *et al.* (1987), Sarkar and Haldar (1990), Mishra and Das (1993), Saha *et al.* (1995 a,b) and Saha and Haldar (1996) as many as 11 species of trichodinid ciliophoran have been recorded from fish in India. They include no new species, but *Paratrichodina notopteri* by Saha *et al.* (1995b).

Trichodinids parasitizing estuarine fish in India are rather poorly known. The only publication (Das *et al.* 1987) on this subject concerns the ecology of animal parasites, including protozoan parasitofauna, of estuarine fishes of West Bengal. In that paper three species of *Trichodina* were recorded from *Liza parsia*, *Lates calcarifer*, *Mystus gulio* and *Glossogobius giuris*, but the authors did not identify these parasites up to species level. During an investigation, between 1995-1997, a new species of *Trichodina* parasitizing *Mystus gulio*, a commercially important estuarine fish of India, was found.

The ciliophoran is described as *Trichodina mystusi* sp. n. in this paper.

MATERIALS AND METHODS

The host fish, *Mystus gulio* (Hamilton) were collected from different parts of Matla River at Canning, about 30 km south of Calcutta. This river regularly receives the water from the Bay of Bengal during high tide. Gill smears were made from freshly captured fishes. Smears with trichodinid ciliophorans were air-dried and stained with Klein's dry silver impregnation technique. Examination of preparations were made under the Olympus phase-contrast microscope, model CH-2. All measure-

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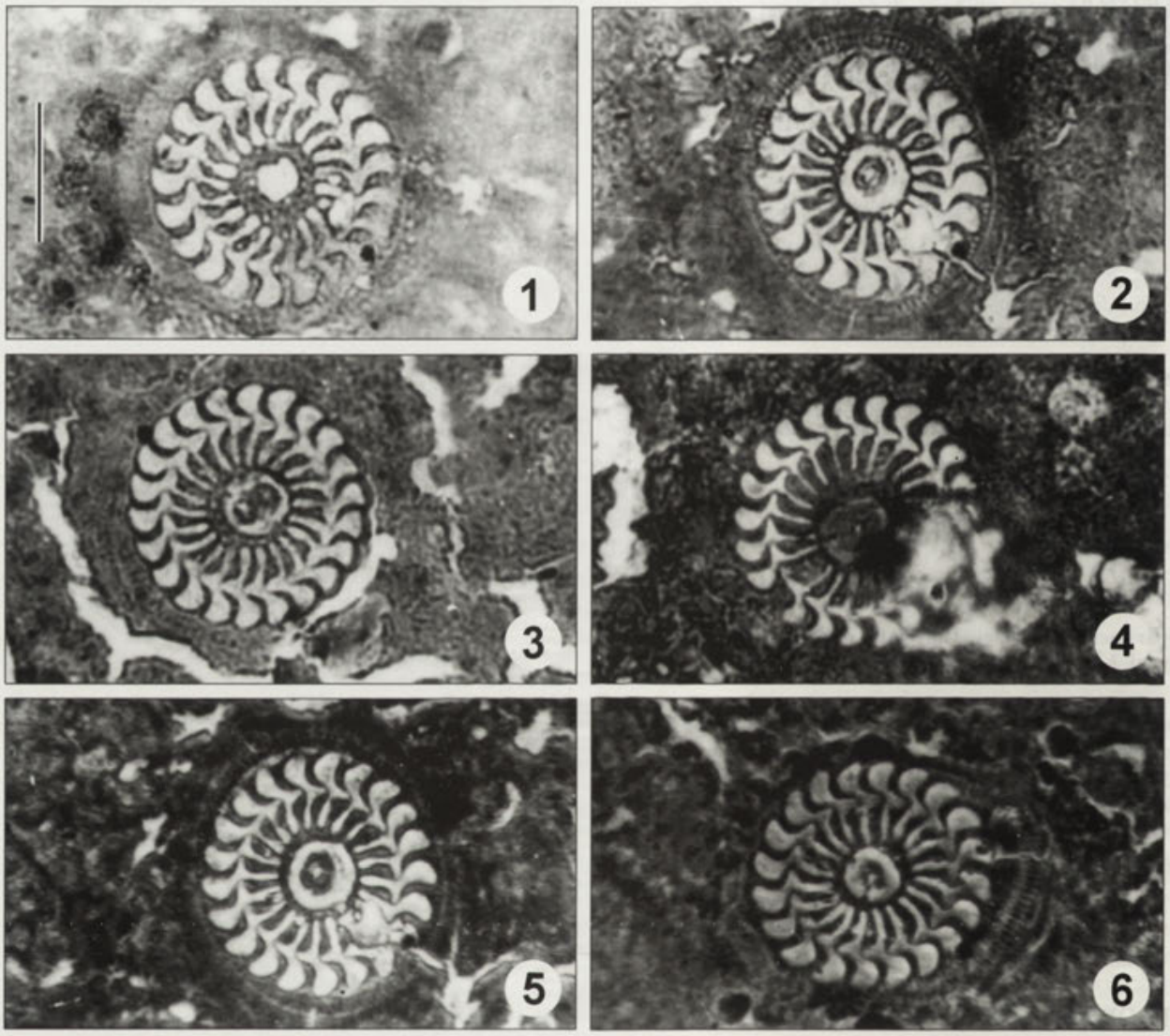


Figs. 1-6. Photomicrographs of *Trichodina mystusi* sp. n. from *Mystus gulio* showing the variation in the structure of the adhesive disc. 1-4 - under light microscope; 5, 6 - under phase-contrast microscope to show the elevation of the central area. Scale bar - 20 µm

ments given in the result are in micrometers. The range is given, followed in parentheses by the arithmetic mean and standard deviation. The body diameter is measured as the adhesive disc plus the border membrane. Numerous photographs were made in order to have a comprehensive morphological analysis of the ciliophoran. Lom (1958), Wellborn (1967) and Van As and Basson (1992) were followed to describe details of the various parts of the adhesive disc.

RESULTS

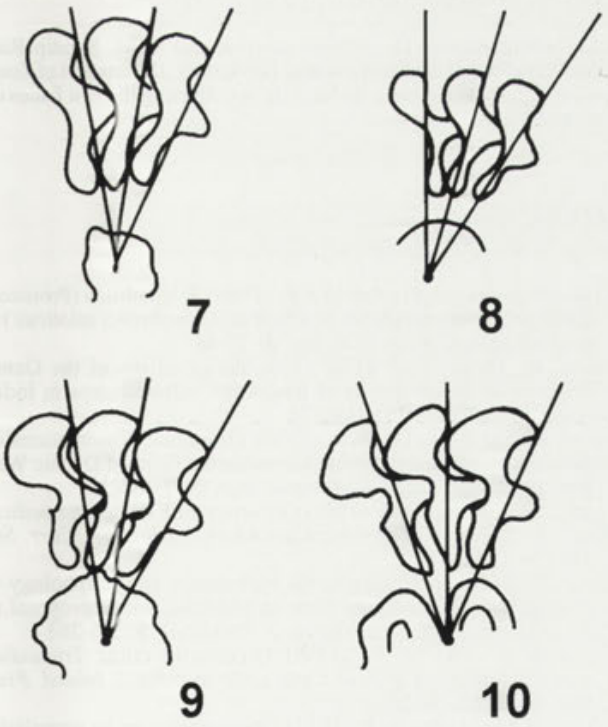
Trichodina mystusi sp. n. (Figs. 1–6)

Description: the body of this trichodinid ciliophoran is small in size having a disc to bell-shape measuring 27.6-36.8 (30.9±2.7) µm in diameter. The adhesive disc is saucer-shaped, 21.4-30.6 (25.0±2.7) µm in diameter,surrounded by a faintly striated border membrane, 1.5-4.1 (2.9±0.5) µm wide. The centre of the adhesive disc is velasquezae type/circle within a circle/ring within a ring type, rarely clear and without notched outline, encircled by a heavily impregnated ring, 5.1-9.2 (6.8±1.0) µm in diameter. In most of the young specimens the central area is dark or subdivided, rarely with a single undivided circle. Diameter of the denticulate ring is 13.3-18.4 (15.2±1.8) µm consisting of 20–24 denticles.

The blade is broad and angular. The distal surface of blade is rounded, but truncated in younger ones, with sharp curve to the anterior surface. The tangent point is

Table 1. A comparison of *Trichodina mystusi* sp. n. and *Trichodina velasquezae* Bondad-Reantaso and Arthur, 1989; measurements in µm

Species	*Trichodina mystusi* sp. n. n = 20		*Trichodina velasquezae* n = 17	
Host	*Mystus gulio*		*Orechromis niloticus*	
Localisation	Gills		Skin, gills	
Locality	Matla River, Canning, West Bengal, India		Leganes, Iloilo, Philippines	
Reference	Present paper		Bondad-Reantaso and Arthur (1989)	
Diameter of the body	27.6 - 36.8	(30.9 ± 2.7)	27.0 - 35.0	(29.9 ± 2.3)
of the adhesive disc	21.4 - 30.6	(25.0 ± 2.7)	22.0 - 29.0	(26.0 ± 2.3)
of the denticulate ring	13.3 - 18.4	(15.2 ± 1.8)	19.0 - 25.0	(22.4 ± 2.1)
of the central area	5.1 - 9.2	(6.8 ± 1.0)	–	
of the clear area	3.0 - 7.1	(5.5 ± 1.0)	–	
Number of denticles	20 - 24	(21.5 ± 1.1)	19 - 23	(21.0 ± 1.2)
Number of radial pins per denticle	5 - 7	(5.3 ± 0.8)	7 - 8	
Dimensions of denticle				
length of the denticle	2.5 - 5.1	(3.8 ± 0.7)	4.6 - 6.0	(5.2 ± 0.5)
length of the ray	2.1 - 3.6	(2.8 ± 0.5)	2.0 - 6.0	(3.3 ± 1.0)
length of the blade	2.5 - 3.6	(3.1 ± 0.2)	3.0 - 5.0	(3.8 ± 0.7)
width of the central part	1.0 - 3.0	(1.9 ± 0.4)	1.0 - 2.0	(1.2 ± 0.3)
Width of the border membrane	1.5 - 4.1	(2.9 ± 0.5)	1.0 - 2.0	(1.8 ± 0.4)
Adoral ciliary spiral	390 - 400°		–	



Figs. 7-10. Diagrammatic drawings of denticles of trichodinids. 7, 8 - *Trichodina mystusi* sp. n. from India; 9, 10 - *T. velasquezae* Bondad-Reantaso and Arthur, 1989, redrawn from Bondad-Reantaso and Arthur (1989) from the Phillippines

large and blunt, at same level as distal surface. The anterior margin forms smooth curve with no prominent apex, extending slightly more than halfway towards the y+1 axis. There is no blade apophysis. The posterior surface forms shallow crescent with deepest point at same level as apex. The blade connection is well-developed and broad. The posterior projection is absent. The central part is robust, tapering towards sharp rounded, sometimes conical, point and fitting tightly to preceding denticle, extending to half way towards the y -1 axis. The shape of section above and below x axis are similar. The ray connection is very short and broad. The apophysis of ray, when present, is not prominent, anteriorly directed. The indentation in lower central part is absent. The rays are well-developed and slightly curved anteriorly or straight in anterior direction, filling large areas between the y axes. The ray has same thickness along its length but with swollen tip. The central clear area, 3.0-7.1 (5.5 ± 1.0) µm in diameter, is elevated from the rest of the central area and in most cases, contain one or more impregnated ring (Figs. 5, 6). There are 5-7 radial pins per denticle. The adoral zone of cilia forms a spiral of about 390 - 400°.

Type host: *Mystus gulio* (Hamilton).

Type locality : Matla River of Canning, West Bengal, India.

Localisation: gill filaments.

Incidence: 30 out of 165 hosts of *Mystus gulio* had been found to be infected with this parasite, occurring in

mixed infection with other species of *Trichodina* and a species of *Tripartiella* from October to April, mostly in November to January.

Holotype: in slide No. MG-1 prepared on 15.1.1996, in the collection of the Protozoology Laboratory, Department of Zoology, University of Kalyani, Kalyani 741235, West Bengal, India.

Paratypes: in the above mentioned slide and in other slides prepared on different dates.

DISCUSSION

Like all other trichodinids, the described species is also characterised by the morphology of the silver impregnated adhesive disc. Many species of *Trichodina* have somewhat similar size and denticle number and clear or subdivided central area as in this ciliophoran. Among these, *Trichodina partidisci* Haider 1964, *T. puytoraci marisalbi* Stein 1976, *T. tenuidens* Faure - Fremiet 1943 and *T. velasquezae* Bondad-Reantaso and Arthur 1989, show some similarities with the present form.

T. partidisci has a clear central area of the adhesive disc, divided in most cases into two areas of unequal size. Although a divided central area is not uncommon in the present trichidinid, but the two areas, even the areas of a tri–divided central area in younger specimen, are always equal in size. The shape of denticles of *T. partidisci* also differs from those of the described species, the blades being more spatulate and possessing a broadly rounded tip. However, a subdivided central area could also been found in *T. jadranica noblei* Lom 1970, *T. elegans* Stein 1979, *T. cotticamephori* Stein 1979, *T. reticulata* Hirschmann and Partsch 1955 and *T. tenuiformis* Stein 1979, but the appearance of these subdivisions and also their denticle shapes differ to a great extent from those of *Trichodina* species described here. Swollen-tipped ray of denticles are found in *T. tenuidens* and *T. puytoraci marisalbi*, but the total shape of denticle and the appearance of central area of the adhesive disc are quite distinctly different from it.

The ciliophoran under discussion is most closely related to and falls within the same range as *T. velasquezae* (Table 1). The central area in both species divided into variable number of clear areas of irregular shapes and sizes. The important difference, however, concerns the structure of denticles (Figs. 7-10). In both the species the denticle is broad, with rounded anterior margin and slightly concave posterior, tip being bluntly pointed. But the anterior margin of blade in the described trichodinid never bears any notch, which is sometimes present in *T. velasquezae*. The shape of ray is also quite distinct in the two species. In *T. velasquezae*, the ray is short and stout, its tip being usually blunt, appearing pointed occasionally (Figs. 9, 10). In contrast, the blade of denticle of the present species is not as broad and curved as in *T. velasquezae*, so that the posterior margin never forms a deep semilunar curve and the anterior margin never touches the y+1 axis (Figs. 7, 8). The ray is also not so curved, stout and crooked like the latter species, but straight and has the same width throughout the length. In addition, the tip of ray is blunt as well as swollen in most of the populations of the present species, rarely pointed, especially in younger ones. Thus, the very peculiar ray of denticles along with unique central area of the adhesive disc distinguish the described species from other species of *Trichodina* that possesses adhesive discs with clear or subdivided central area. So, we propose to establish this ciliophoran as *Trichodina mystusi* sp. n. after the generic name of the host fish.

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A Study of the Structure and the Life Cycle of *Gymnophrys cometa* Cienkowski, 1876 (Gymnophrea cl. n.) with Remarks on the Taxonomy of the Amoebo-flagellated Genera *Gymnophrys* and *Borkovia*

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Summary. The results of an ultrastructural study of the fine structure of three forms (amoebo-flagellate, zoospore or motile flagellated stage and cyst) of a fresh-water reticulopodiate protist *Gymnophrys cometa* Cienkowski, 1876 are presented. The amoebo-flagellate cells are solitary and move with the help of short lobose pseudopodia which may be found in addition to the reticulopodia. Adjacent to a massive central nucleus there is a single dictyosome and there is a pair of kinetosomes lying almost parallel to each other and having a usual 9-triplet organization. Mushroom-shaped protrusions without any internal substructures and axonemes are associated with each kinetosome and are argued to be reduced flagella. Microtubular or microfibrillar rootlets were not observed. Mitochondrial cristae are plate- or ribbon-like. Reticulopodiate pseudopodia branch and possess bundles of 2-6 longitudinal microtubules. Numerous complex extrusomes (microtoxicysts) occur in the reticulopodia and in the cortical cytoplasm. Cysts are globular and surrounded by a double envelope; their cytoplasm is dark and filled by extensive arrays of endoplasmic reticulum and storage granules. The general morphology of the cell changes very little during transformation from the amoeboid state to the motile state. The flagellated stage has two heterodynamic flagella and an amoeboid outline of the rear part of the cell. A new class Gymnophrea including genera *Gymnophrys* Cienkowski, 1876 and *Borkovia* Mikrjukov *et* Mylnikov, 1996 is proposed; and a taxonomic revision of these two genera is included. The new class is characterised as a group of reticulopodiate amoebo-flagellated protists with lamellar mitochondrial cristae, complex microtoxicysts, a closed pleuromitosis with an extranuclear spindle, possibly having biflagellated cells. The affinities of the class Gymnophrea to other Protista are unclear.

Key words: *Borkovia desaedeleeri* nom. n., Gymnophrea cl. n., *Gymnophrys cometa,* Protista, Protozoa, Rhizopoda.

INTRODUCTION

Recently we (Mikrjukov and Mylnikov 1995; Mikrjukov 1995 b, 1998) have described the fine structure and the biology of *Borkovia cometa* (Penard, 1902) Mikrjukov *et* Mylnikov, 1996 under the name of *Penardia cometa* (Penard, 1902) De Saedeleer, 1934. We showed it to be a naked reticulopodiate rhizopod. We noted similarities with the amoeboid protist *Gymnophrys cometa* Cienkowski, 1876, which has a complex life cycle including states of an amoeba, a cyst and a biflagellated zoospore (Mikrjukov and Mylnikov 1996, 1997). Patterson (1992) and Patterson and Zölffel (1991) have studied organisms referred to by them as *Gymnophrys* (but which are identical to *Borkovia*: see Fig. 1f) and have found complex concentric extrusomes and two reduced flagella. *Borkovia*

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cometa has mitochondria with lamellar cristae, branching reticulopodia with longitudinal bundles of 2-6 microtubules, complex extrusomes which we refer to as microtoxicysts (Mylnikov 1988), and two reduced flagella arising from a pair of conventional almost parallel kinetosomes. The nuclei of *B. cometa* divide by closed pleuromitosis with an extranuclear spindle similar to that in Parabasalia. The cells of *Borkovia* are immobile and form chain- or ring-like aggregations which adhere to the substrate, by means of complex cell contacts (Mikrjukov 1998).

Taxonomic history

Cienkowski (1876) described a naked reticulopodiate amoeboid organism under the name of *Gymnophrys cometa*. It had numerous granules at its reticulopods (Fig. 1 a). A few decades later Penard (1902) described a different species of the filopodiate amoeba under the name *G. cometa*. Penard's protist (Fig. 1 b) differed from Cienkowski's amoeba because it has not anastomosing axopods, and Penard did not comment on the presence of granules. Later, De Saedeleer (1934) redescribed Penard's rhizopod (Fig. 1c). He also described an organism with a cell body and fine branching but not anastomosing pseudopodia and no granules were mentioned, and transferred it to the genus *Penardia* Cash, 1904. However, De Saedeleer retained the species name 'cometa' referring to it as *Penardia cometa* (Penard, 1902) De Saedeleer, 1934. This author placed the genus *Penardia* in a new order Aconchulinida of the class Filosea Leidy, whereas the genus *Gymnophrys* was placed in the order Athalamida Haeckel in a new class Granuloreticulosea. This placement was accepted by Deflandre (1953), Bovee (1985a, b) and De Puytorac *et al.* (1987). Doflein (1916) and Kudo (1954) considered both organisms as members of the rhizopod order Proteomyxida Lankaster (an ill-defined group of amoebae distinguished by having radiating pseudopods) and the family Vampyrellidae Doflein (proteomyxids without a flagellated swarmer). *Gymnophrys* was considered by Doflein as a junior synonym of *Biomyxa* Leidy since the revision by Rhumbler (1903). Page (1991) placed both genera in the order Athalamida Haeckel of the Class Granuloreticulosea De Saedeleer because of similarities of pseudopodial morphology. Patterson (1994) did not recognise the proteomyxids nor they aconchulinids, and placed both *Gymnophrys* and *Penardia* in a group of protists of uncertain affinities. We (Mikrjukov and Mylnikov 1996) have separated Penard's organism (i.e. *Penardia cometa sensu* de Saedeleer) from the genus *Penardia* Cash in a new monotypic genus *Borkovia* on the base of the presence of reticulopodia bearing granules (extrusomes). We have proposed a new family Gymnophryidae Mikrjukov *et* Mylnikov, 1996 (Protista *incertae sedis*) uniting *Gymnophrys* and *Borkovia*. However, its species name *B. cometa* is still incorrect because the basionym remains in use for *Gymnophrys cometa*. We here offer a new name.

The present work is a study of the fine structure and biology of *Gymnophrys cometa*. In view of the exceptional similarity between the amoeboid states of *Borkovia* and *Gymnophrys*, we concentrate on the differences between these two organisms - the polymorphic characteristics of *G. cometa* (e.g. the morphology of its cysts and zoospores), and on the relationships of gymnophryid rhizopods to other Protista.

MATERIALS AND METHODS

Gymnophrys cometa was isolated from the waste treatment plants Borok (Yaroslavl province, Russia) and cloned. The amoebae were cultivated in Petri dishes in Pratt medium containing *Enterobacter aerogenes*. Cysts are always present in the cultures with an abundance about one third that of the amoebae. Flagellated stages are normally absent; but can be obtained by agitating the cultures with a magnetic stirrer.

For electron-microscopic studies, living organisms were harvested by centrifugation and fixed with a mixture of 2% osmium tetroxide and 1.2% glutaraldehyde in 0.05 M cacodylate buffer for 15-30 min at 1°C. After dehydration in a series of alcohols and water-free acetone, the cells were embedded in a mixture of Epon 812 and Araldite M. The sections were stained with uranyl acetate and lead citrate and photographs taken with a JEM-100 B microscope.

RESULTS

Light microscopy

Amoeboid cells of *Gymnophrys cometa* (Figs. 1 a, g-i, o) are common in the cultures. In contrast to those of *Borkovia cometa* (Figs. 1 b-f), they do not aggregate and are mobile. They can move with the help of short lobopods (Fig. 1 o, arrows) which occur alongside usually 3-5 non-anastomosing reticulopodia. Extrusomes are easily seen on the reticulopodia as large granules. Both reticulo- and lobopodia form and are resorbed at any part of the cell surface. The pseudopodia change their shape and direction of growth; so the general outline of each organism varies considerably and can take on a radiating form (Fig. 1 g). The nucleus in the living cell is normally obscured by the cytoplasm. Several contractile vacuoles

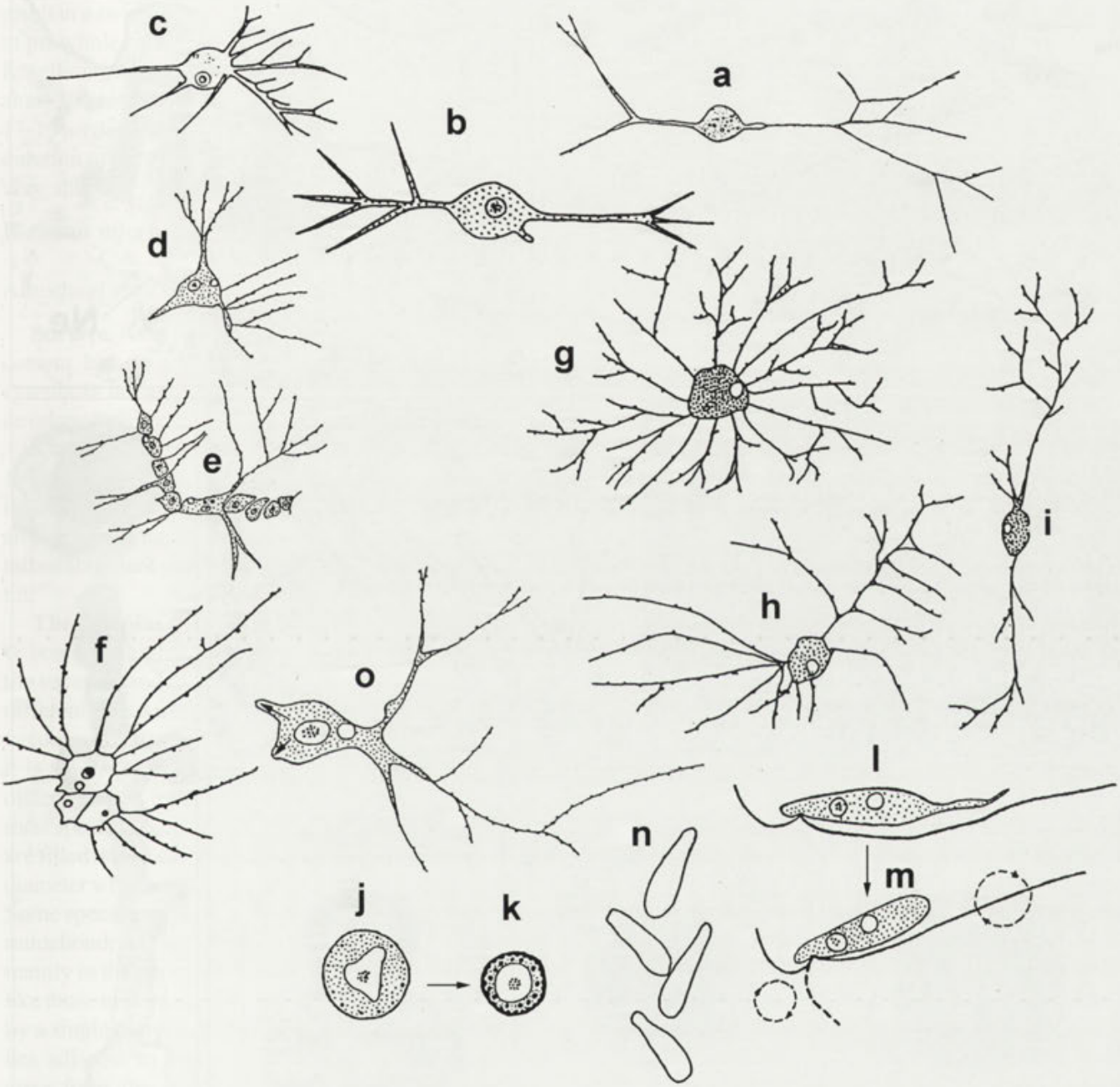
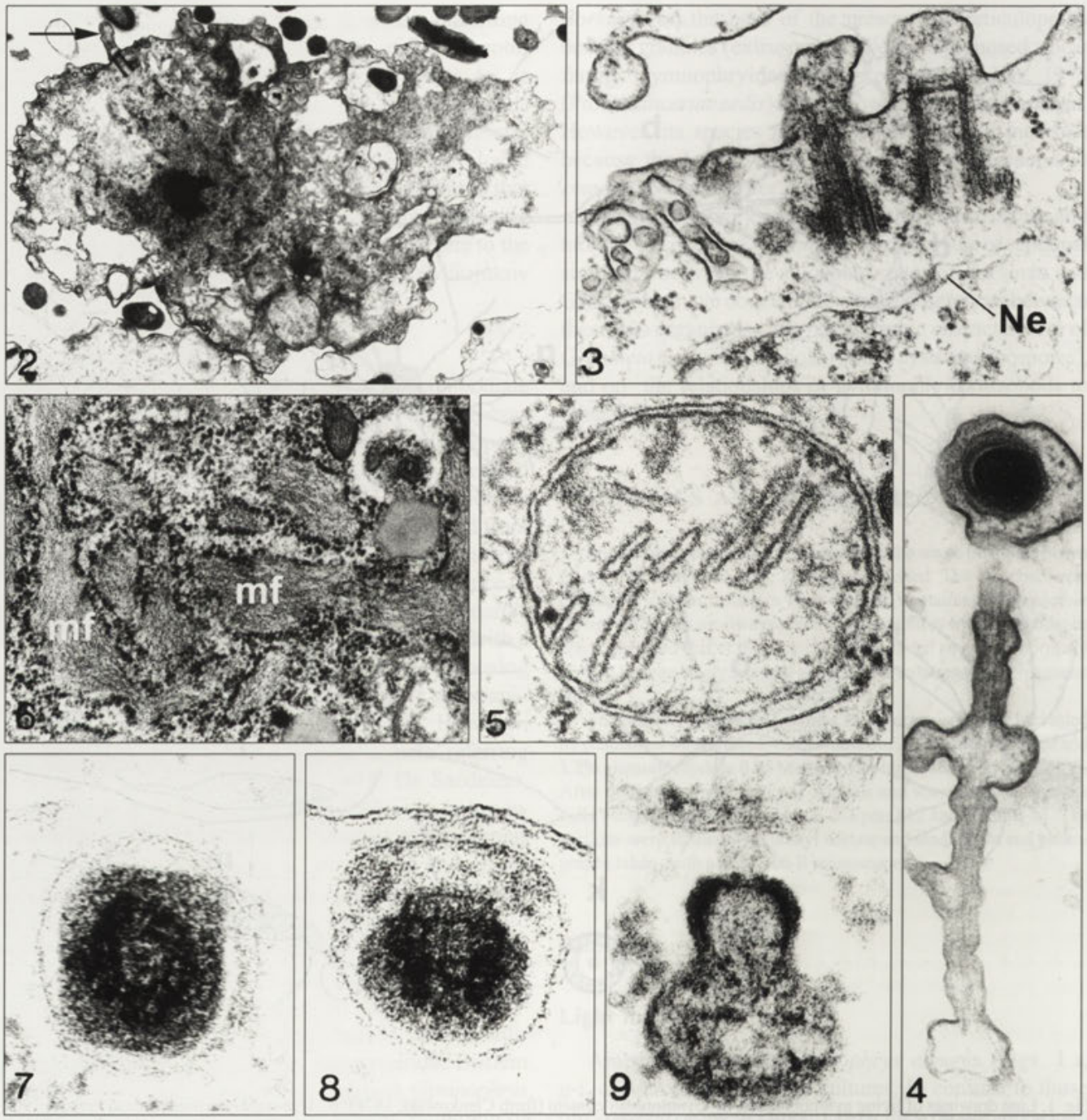


Fig. 1. Line drawings of living gymnophryids. a - *Gymnophrys cometa* (from Cienkowski, 1876); b - *Gymnophrys cometa* sensu Penard, 1902 (after Penard, 1902); *Penardia cometa* (after De Saedeleer, 1934); d,e - individual cell and cell aggregation *Borkovia cometa* (our observations); f - cell aggregation of *Borkovia cometa* observed by Patterson (1992); g,h - amoeboid cells of *Gymnophrys cometa* (present observations); j,k - incysting stage and cyst of *G. cometa*; l,m - stage of transformation to zoospore and mature zoospore of *G. cometa*; n - the mode of swimming of zoospore of *G. cometa*; o - crawling specimen of *G. cometa* (arrows indicate lobopods)

occur at different sites of the body (*B. cometa* has only one contractile vacuole). The diameter of the cell body of the amoebae is 5-7 µm, the length of reticulopodia can exceed 3-4 times the cell body diameter.

Cysts are always present in the culture along with encysting and excysting forms. During the encystment, the cell becomes rounded, its diameter decreases (Figs. 1 j, k), a lot of large storage (polysaccharide?) granules appear in the peripheral cytoplasm, and an envelope appears at its surface. Mature cysts are 2.5-3.5 µm in diameter, opaque and highly refractile because of the storage granules in the external cytoplasm.



Figs. 2-9. General morphology of the amoebo-flagellate stage of *Gymnophrys cometa*. 2 - section through the whole cell, mushroom protrusion-arrow, x 11 400; 3 - pair of kinetosomes lies close to the nuclear envelope (Ne), electron dense material is visible around the basal parts of kinetosomes, x 80 000; 4 - distal end of reticulopodium, bundle of several longitudinal microtubules and extrusome are seen, x 60 000; 5 - mitochondrium with flat, ribbon-like cristae, x 120 000; 6 - fragment of the peripheral lacunar system containing microfibrillar material (mf), x 40 000; 7-9 - cross and longitudinal sections through an extrusive organelles - microtoxicysts and discharged organelle, x 200 000

Transformation into a flagellated stage was observed only from the amoeboid state (Figs. 1 l, m). During transformation, two heterodynamic flagella appear at the front of the cell, its rear retains reticulopodia or has an amoeboid outline even in the mature swarmer. The nucleus is still hard to see, a single contractile vacuole is well seen. The front flagellum is shorter (3-5 μm) than the other flagellum and beats with rotating movements which

result in a swinging motion of its fore-part and the rotation of the whole cell during swimming (Fig. 1 n). A recurrent flagellum may be 20-25 μm long and extends backwards along the cell body. The cell body has an oblong form, is 13-15 μm long and 3-4 μm in diameter at its rear part. The duration of the flagellated state in the culture seems to be very short.

Electron microscopy

Amoeboid state

Surface. As in *Borkovia*, the cell surface in *Gymnophrys cometa* has no structural modifications in the cortical cytoplasm nor any external structures except for a poorly developed glycocalyx.

The nucleus is of the vesicular type (*sensu* Raikov 1982), 1.5-3.0 μm in diameter. In contrast to *Borkovia*, it has an irregular shape (Fig. 2). Its centre is occupied by an extensive nucleolus, about 1.0 μm in diameter; with rather abundant peripheral aggregates of heterochromatin.

The cytoplasm and its organelles. The cytoplasm of *G. cometa* is highly vacuolized, containing many contractile vacuoles and many digestive vacuoles with bacteria at different stages of digestion, and also numerous structural vacuoles containing small membranous vesicles (Fig. 2); it is similar to those observed in *Borkovia*. There is no differentiation into the ectoplasm and the endoplasm. In most specimens, some regions of the peripheral cytoplasm are filled with a complex system of lacunae 0.2-0.4 μm in diameter which contains a microfibrillar secretion (Fig. 6). Some specimens do not have a lacunar system. Spherical mitochondria (Fig. 5), 0.4-0.5 μm in diameter, are found mainly in the peripheral cytoplasm; they have flat cristae like those in *Borkovia*. The Golgi apparatus is represented by a single dictyosome consisting of several cisternae. It lies adjacent to the nucleus. Numerous coated vesicles arise from the external nuclear membrane and move towards the dictyosome. Others arise from the dictyosome and appear to move to the plasma membrane. Contractile vacuoles are numerous, they form in different regions of the cell and can fuse with the plasma membrane at any location; their size normally does not exceed 0.7 μm.

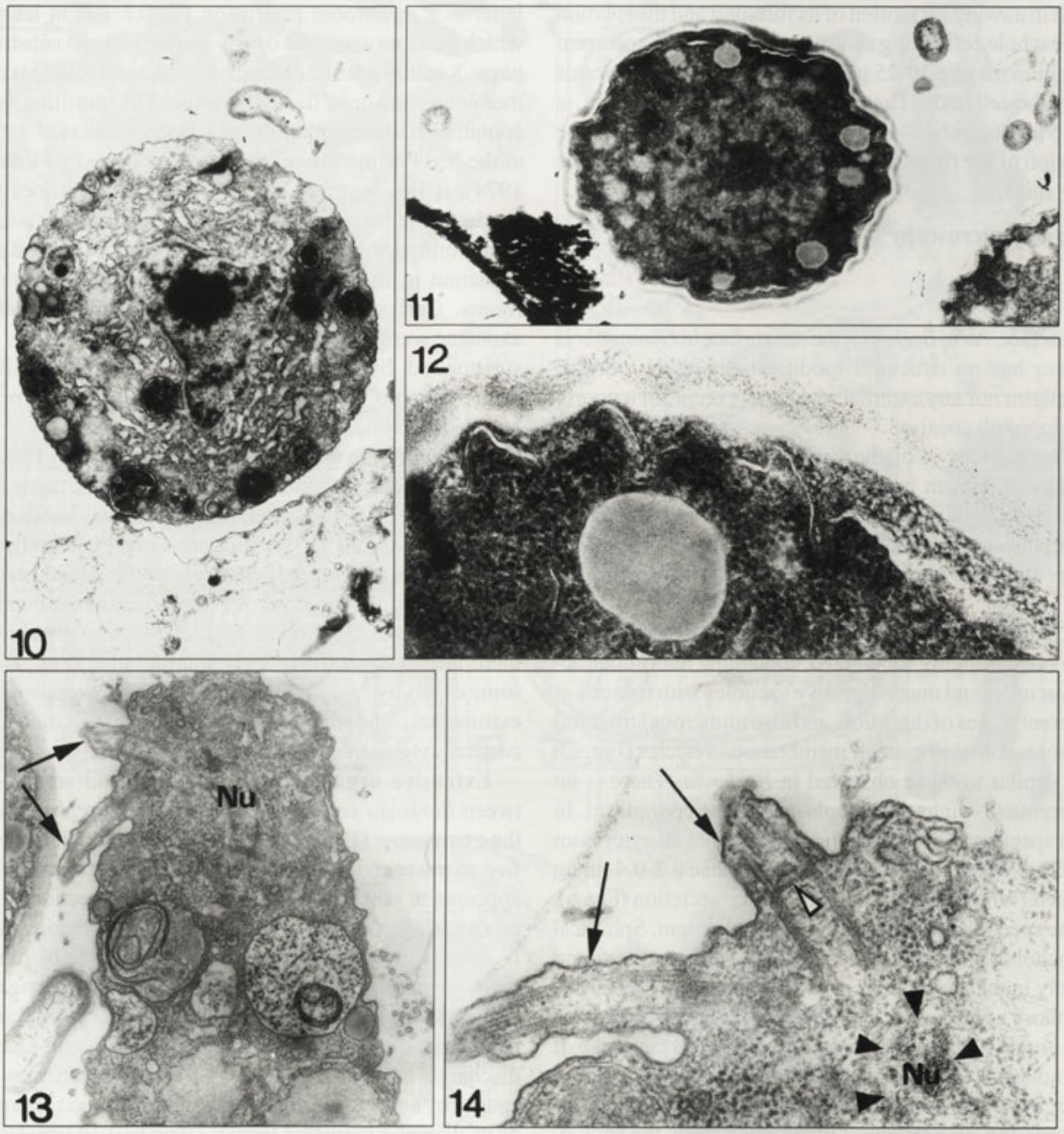
Flagella. The flagellar apparatus (Figs. 2, 15 A) is identical to that of *Borkovia*. It includes a pair of normally developed (Grain *et al.* 1988, Andersen *et al.* 1991) kinetosomes lying almost in parallel (*ca.* 0.4 μm in length and 0.2 μm in diameter). Each kinetosome gives rise to a short highly reduced flagellum (Figs. 2 arrow, 15 A). The latter is a mushroom protrusion (*ca* 0.6 μm in length) which lacks an axoneme or any visible internal substructures. Kinetosomes lie immediately adjacent to the plasma membrane and near the dictyosome. The transition zone contains a transition plate (Type 1*a* after Grain *et al.* 1988) at the level of the plasma membrane (Type 1 of Pitelka 1974); it lies entirely within the lumen and does not reach the plasma membrane (Fig. 15 A). The *a*- and *b*-microtubules of the kinetosomes extend beyond the transition plate and end in the basal part of the mushrooms. Central pairs of microtubules in the flagellar axoneme were not observed. The bases of kinetosoms are surrounded by amorphous electron-dense satellites (Fig. 3), but no microtubular nor microfibrillar elements of a rootlet system were observed.

Reticulopodia are thin and highly branching. They so not have broadened bases as in *Borkovia* (Mikrjukov and Mylnikov 1995). They contain longitudinal bundles of 2-6 microtubules (Fig. 4) which arise in the cell body. No MTOCs were observed. Where reticulopodia branch there are expanded regions of cytoplasm, and the bundles of microtubules also divide. At their the most distal parts there are always at least two microtubules (Fig. 4). In some expanded regions of the reticulopodia there are large extrusomes, and these may also be observed in the cortical cytoplasm too (Fig. 4).

Extrusive organelles. An important difference between *Borkovia* and *Gymnophrys* is the morphology of the extrusomes (Figs. 7-9, 16). Although both are classified as microtoxicysts (Mylnikov 1988) with a concentric appearance when observed in transverse section, their modes of ejection are quite different. Microtoxicysts of *G. cometa* average 0.15 μm in length and 0.14 μm in diameter while in *B. cometa* they are *ca* 0.16 μm long and 0.20 μm wide. Inside the organelle there is a massive axial element, and is enclosed within an apical enfolding. During discharge internal cylinder in everted, which may be caused by an increase of pressure within the axial element as its diameter increases from 0.10 up to 0.14 μm during discharge. During the ejection, a microgranular material is extruded from the axial element.

Cysts

During encystment, the nucleus takes a more regular outline, the cell becomes more spherical and is *ca* 4.0 μm in diameter. Pseudopodia and flagella are withdrawn. There is a dramatic increase of rough endoplasmic reticulum through the whole cytoplasm (Fig. 10), and a lot of granules appear in the peripheral region of the cell. As encytsment continues, the nucleus becomes central, the



Figs. 10-14. Encystment stage, cyst and motile biflagellated form of *Gymnophrys cometa*. 10 - beginning of encystment, x 14 300; 11,12 - mature cyst and fragment of its peripheral part (a folded shape of the plasma membrane and the bilayer external envelope are visible), x 14 300 and x 68 600, respectively; 13,14 - biflagellated form of *G. cometa* showing position of the nucleus (Nu) and two flagella (arrows); 14 - nuclear envelope (black arrowheads), transition plates in the axial flagellum (white arrowhead), x 21400 and x 57100, respectively

cytoplasm becomes condensed, and the number of storage granules increases (Fig. 11). Then the plasma membrane becomes folded and a two-layered envelope is excreted on its surface. The internal layer is less electron dense than the external layer (Fig. 12). The organelles of the cytoplasm are hard to see, and we were unable to determine if kinetosomes remain in mature cysts. During the excystment, the cyst envelope breaks and begins to

break away from the plasma membrane, while the cell increases in size. The cytoplasm becomes more transparent, the 'storage granules' in its peripheral regions decreasing and resolving of supply granules and a presence of a pair of kinetosomes is seen. On the whole, all processes are going in the order, which is reverse to that during the incystment.

Motile stage

The spatial relationships between cytoplasmic organelles in *Gymnophrys* change very little during transformation from the amoeboid state to the motile state (Figs. 13, 14). The nucleus and a single dictyosome move to the front of the cell, where the two flagella form. The angle between kinetosomes increases until they stand almost perpendicular to each other. Transition plates extend from the lumen of the kinetosome to the plasma membrane (Figs. 14 white arrowhead, 15 C). The pair of kinetosomes lie beside the dictyosome. No roots were observed nor were any modifications of the cortical cytoplasm or of the cell surface. Most of the cell remains amoeboid and extrusomes may be seen rarely. The cytoplasm is filled with numerous small and large vacuoles with various contents (often with small membrane-bounded vesicles); there are some digestive vacuoles although the phagocytosis is not observed in the motile flagellates (digestive vacuoles therefore presumably remaining from the amoeboid phase).

DISCUSSION

We conclude that *Borkovia cometa* (Penard) Mikrjukov *et* Mylnikov, 1996 and *Gymnophrys cometa* Cienkowski, 1876 represent a well defined of reticulopodiate amoeboflagellates. The group differs from other protists with thin branching pseudopodia with extrusomes, by the flat shape of mitochondrial cristae, complex microtoxicysts (forming in ER), the type of the mitosis and some other characters.

The question on the evolutionary relationships position of these organisms remains unresolved. However flat cristae of mitochondria together with the presence of zoospores separate these two genera from the class Granuloreticulosea, because all previously studied members of the latter are characterised by tubular cristae (Levine *et al.* 1980; Page 1987, 1991). The same reason prevent us from placing of gymnophryids in the class Sarcomonadea Cavalier-Smith (1993, 1995), where he placed many amoebo-flagellate taxa. The flat mitochondrial cristae suggest these organisms most probably are related to another branch of the evolutionary tree of Protista (Taylor 1976; Patterson 1988, 1994; Patterson and Brugerolle 1988; Cavalier-Smith 1993, 1995; Corliss 1994; Cavalier-Smith and Chao 1995). O'Kelly (1993) regards the jacobids showing all kinds of cristae as a group placing at the base of the protistan evolutionary tree, however this decision and the presence of jacobids as a natural group is rather doubtful.

We recently (Mikrjukov and Mylnikov 1996) proposed that *Gymnophrys* and *Borkovia* be placed in separate family Gymnophryidae Mikrjukov *et* Mylnikov (Protista *incertae sedis*); it becomes obvious now on the base of the adduced data, that the range of this taxon ought to be considerably higher and be equal to that of the class Granuloreticulosea.

Gymnophrea cl. n.

Diagnosis: reticulopodiate amoeboid protists, constantly with a pair of short reduced flagella with axonemes arising from a pair of almost parallel kinetosomes. Mitochondrial cristae are flat, plate- or ribbon-like. Reticulopodia contain a longitudinal bundle of 2-6 microtubules. Complex extrusive organelles have a concentric structure. Gymnophrea are polymorphic with an amoebo-flagellate stage, a cyst and biflagellated stage with naked heterodynamic flagella. Nuclear division is a closed pleuromitosis with an extranuclear spindle.

Two genera: *Gymnophrys* Cienkowski, 1876, and *Borkovia* Mikrjukov *et* Mylnikov, 1996, in one family Gymnophryidae Mikrjukov *et* Mylnikov, 1996.

Genus *Gymnophrys* Cienkowski, 1876, emend.

Diagnosis: solitary amoeboid protists with branching and sometimes anastomosing reticulopodia possessing numerous extrusomes. Amoeboid are able to move with the help of short lobopods. The massive central nucleus has a large nucleolus. Polymorphic with an amoeboflagellate (basic) stage, a cyst and a biflagellated form with heterodynamic flagella lacking mastigonemes. An amoeboflagellate stage possesses two - reduced flagella.

Monotypic. The type species - *G. cometa* Cienkowski, 1876 (Fig. 1 d).

Genus *Borkovia* Mikrjukov *et* Mylnikov, 1996

Diagnosis: aggregated or solitary amoeboid protists with branching reticulopodia possessing numerous extrusomes. Cells are immobile but can change their outline resulting from flowing of the cytoplasm of reticulopodia. A massive central nucleus has a large central nucleolus. Each cell possesses two short mushroom protrusions - reduced flagella.

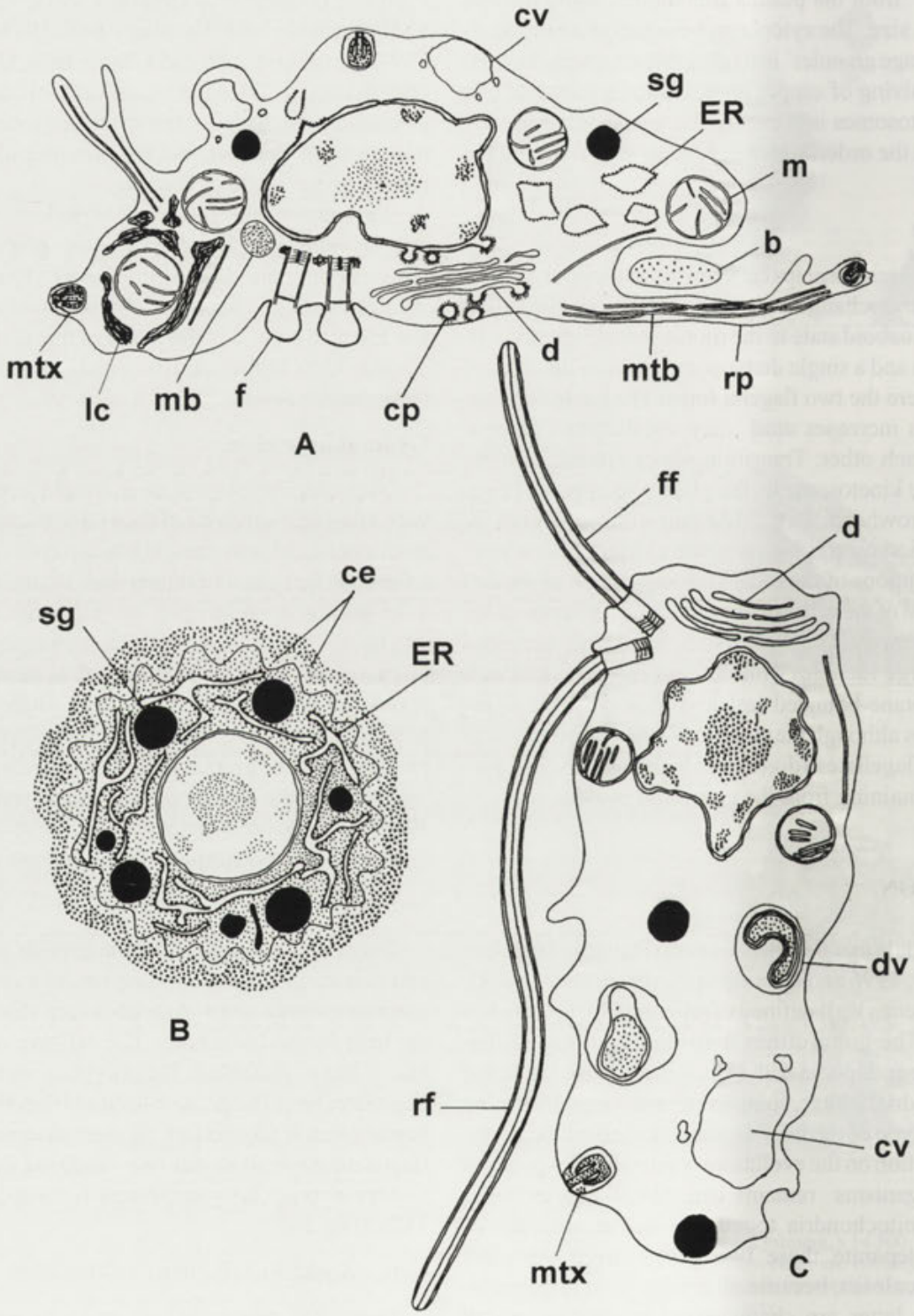


Fig. 15. Diagrams of three stages of the life cycle of *Gymnophrys cometa*: A - amoebo-flagellate form , B - the cyst, C - motile flagellated form. b - bacterium; ce - cyst envelope; cp - "coated" pit; cv - contractile vacuole; d - dictyosome; dv - digestive vacuole, ER - endoplasmic reticulum; f - reduced flagellum of the amoebo-flagellate state; ff - frontal flagellum of zoospore; lc - microfibrillar lacunar system, m - mitochondrium; mb - microbody-like organelle; mtb - microtubules; mtx - microtoxicyst; rf - recurrent flagellum of zoospore; rp - reticulopodium; sg - storage granule

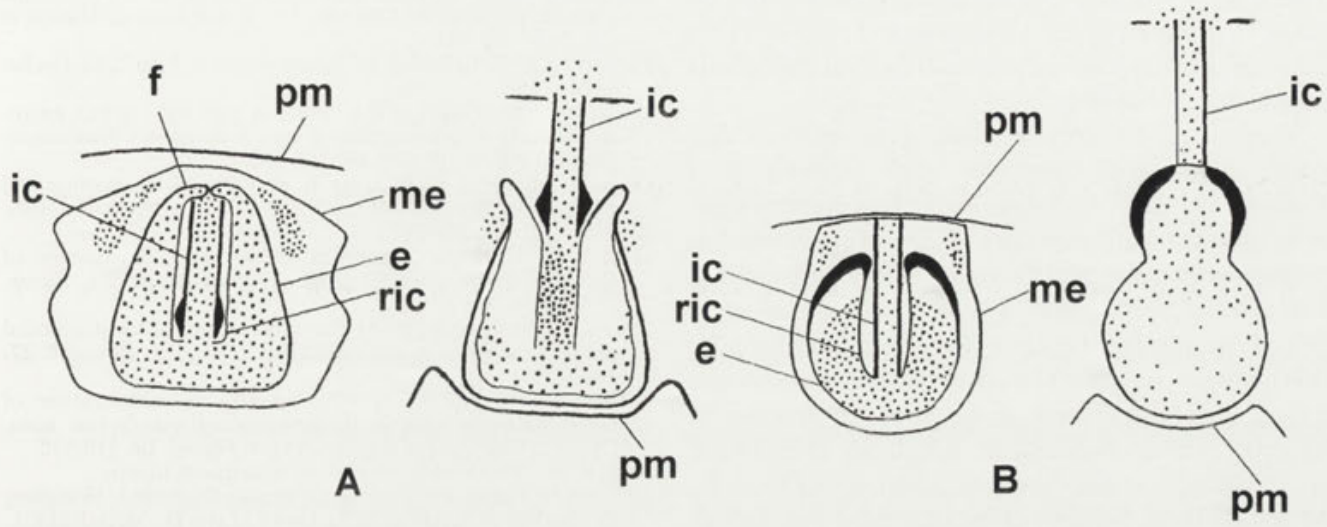


Fig. 16. Passive and discharge states of extrusive organelles of *Borkovia desaedeleeri* (A) and *Gymnophrys cometa* (B) (from Mikrjukov and Mylnikov 1996). e - envelope of axial element; f - fold; ic - internal cylinder; me - membrane of extrusome; pm - plasma membrane; ric - reservoir of internal cylinder

Extrusomes are microtoxicysts. Cysts and zoospores are unknown.

Monotypic. The type species - *B. desaedeleeri* nom. n.

The species name *Borkovia cometa* (Penard, 1902) Mikrjukov *et* Mylnikov, 1996 is changing by a new one; the description of this species is following:

Borkovia desaedeleeri nom. n. (Figs. 1 b-f)

Synonyms: *Penardia cometa* (Penard, 1902) De Saedeleer, 1934; *Borkovia cometa* (Penard, 1902) Mikrjukov *et* Mylnikov, 1996)

Diagnosis: freshwater reticulopodiate amoeboid protists, 5.0-6.6 μm (8.5-30 μm after De Saedeleer 1934) in diameter, having two reduced flagella as mushroom protrusions, about 1 μm in length. Reticulopodia are granular, with aggregates of cytoplasm around extrusomes. With central oval nucleus oval, about 2.4 μm in diameter, with a large central nucleolus, about 0.7 μm in diameter. Cells usually not solitary but forming chains- or ring-like aggregations of 5-10 cells resulting from incomplete cell divisions.

Etymology: the species name honours H. De Saedeleer. This is just a new name not a new species. Any reference to type must refer to the original description in Penard, 1902.

The existence of the class Gymnophrea does not allow us to make any conjectures on whether all members of the order Athalamida Haeckel [which previously included genera *Gymnophrys* and *Penardia* (Page 1991)] should be transferred to this new class. No more athalamids have been studied by electron microscopy, and neither cysts nor zoospores are observed in any other species. Electron - microscopical investigations of species of the genus *Biomyxa* Leidy and the type-species of the genus *Penardia* Cash - *P. mutabilis* Cash are necessary for further conclusions on the position of athalamids in the system of Protista.

We are unable to determine the position of the class Gymnophrea in the general system of Protista. Other organisms with flat mitochondrial cristae include the centrohelid heliozoa (Centrohelida Kühn). Both groups have: (i) flat (similar to ribbon-like) mitochondrial cristae, (ii) complex extrusomes originating in the ER (Mikrjukov 1995 a, b) and with a concentric transverse structure, (iii) pseudopods with a supporting bundle of several microtubules. With the exception of a single record by Schaudinn (1896) of a biflagellated swarmer of the centroheliozoon *Choanocystis aculeata* (which has not been confirmed by numerous later investigations of centrohelids) there is no record of a flagellated stage in the centrohelids. The centrohelids have a clear identity but (Davidson 1972, Bardele 1975, Dürrschmidt and Patterson 1987) are also of uncertain affinities. However conjecture that gymnophryids may be a group related to the flagellate ancestor of the centroheliozoa does not make things clear in the question on the position of these two groups in the

system as a whole. Another lamellicristate rhizopod group - the proteomyxids (Anderson and Höffler 1979) share significantly less similarity with the gymnosphaerids than do the centrohelids.

There is a similarity between gymnophryids and some other amoebo-flagellates, such as *Massisteria* (Cercomonadida) or *Chlorarachnion*, however this similarity is based mainly on the presence of reticulopodia, complex extrusomes, and the ability to form biflagellated (Hibberd and Norris 1984, Mylnikov 1986 a, Grell *et al.* 1990, Patterson and Fenchel 1990, Grell and Schüller 1991). Microtoxicysts of *G. cometa* are similar in structure to those of some members of the genus *Cercomonas* of the order Cercomonadida, in which an eversion of an internal cylinder also takes place (Mylnikov 1986 a, b). We regard these similarities as superficial because all organisms mentioned above have tubular mitochondrial cristae. We accept the view that the shape of mitochondrial cristae is conserved (Taylor 1976,1978) and that these organisms belong to another branch of the evolutionary tree of eukaryotes (Patterson 1988,1994; Cavalier-Smith 1993); extrusion of the microtoxicysts of *Borkovia* does not involve eversion, but the internal cylinder simply slides out (Fig. 16 A).

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