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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

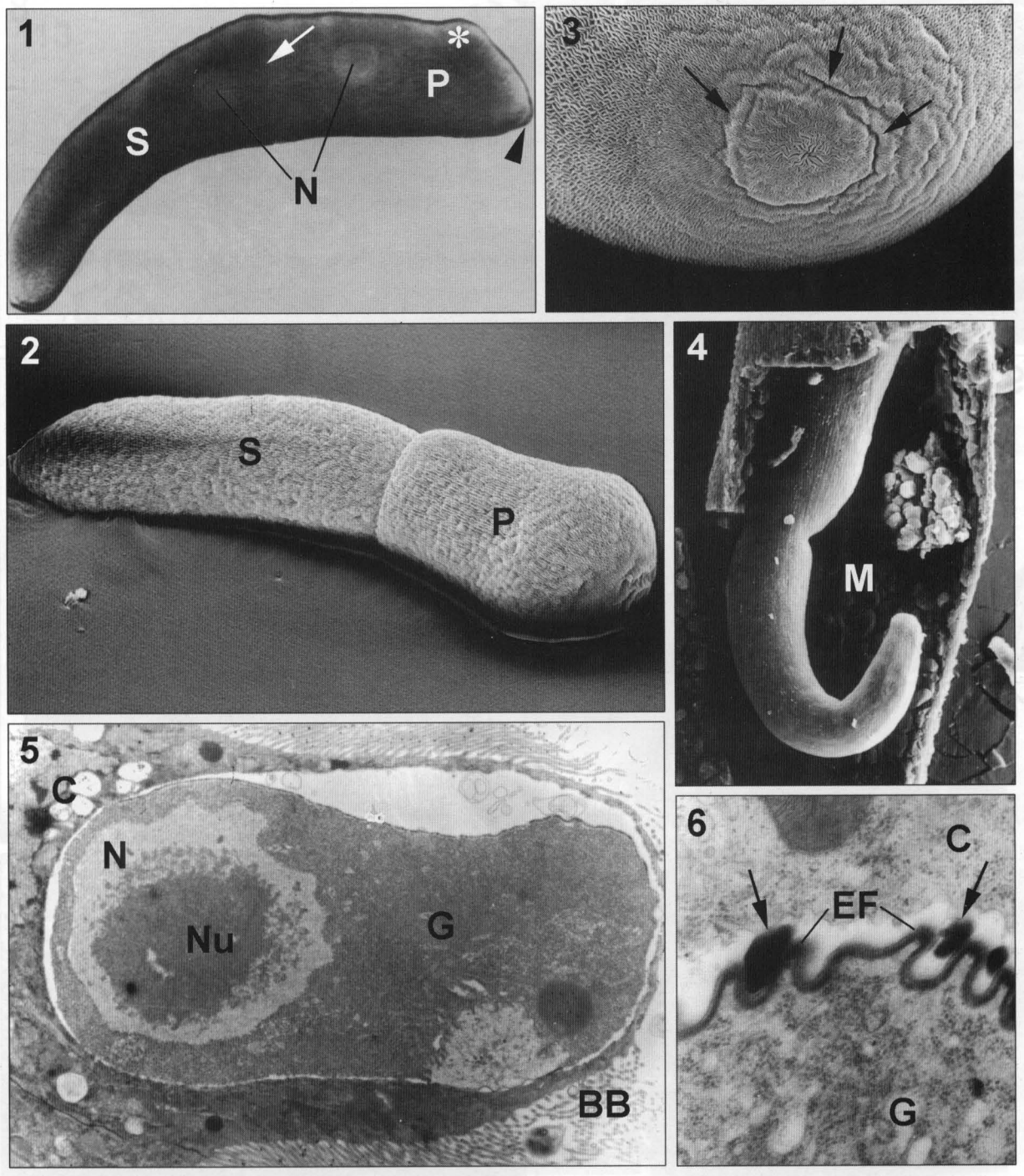
The midgut of some nymphs of *Baetis rhodani* is the site of development of gregarines belonging to *Enterocystis racovitzai*. This parasite, which shows the early pairing of the individuals to form syzygies, causes heavy infection due to the remarkable amount of gregarines into the mayfly midgut lumen. 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).

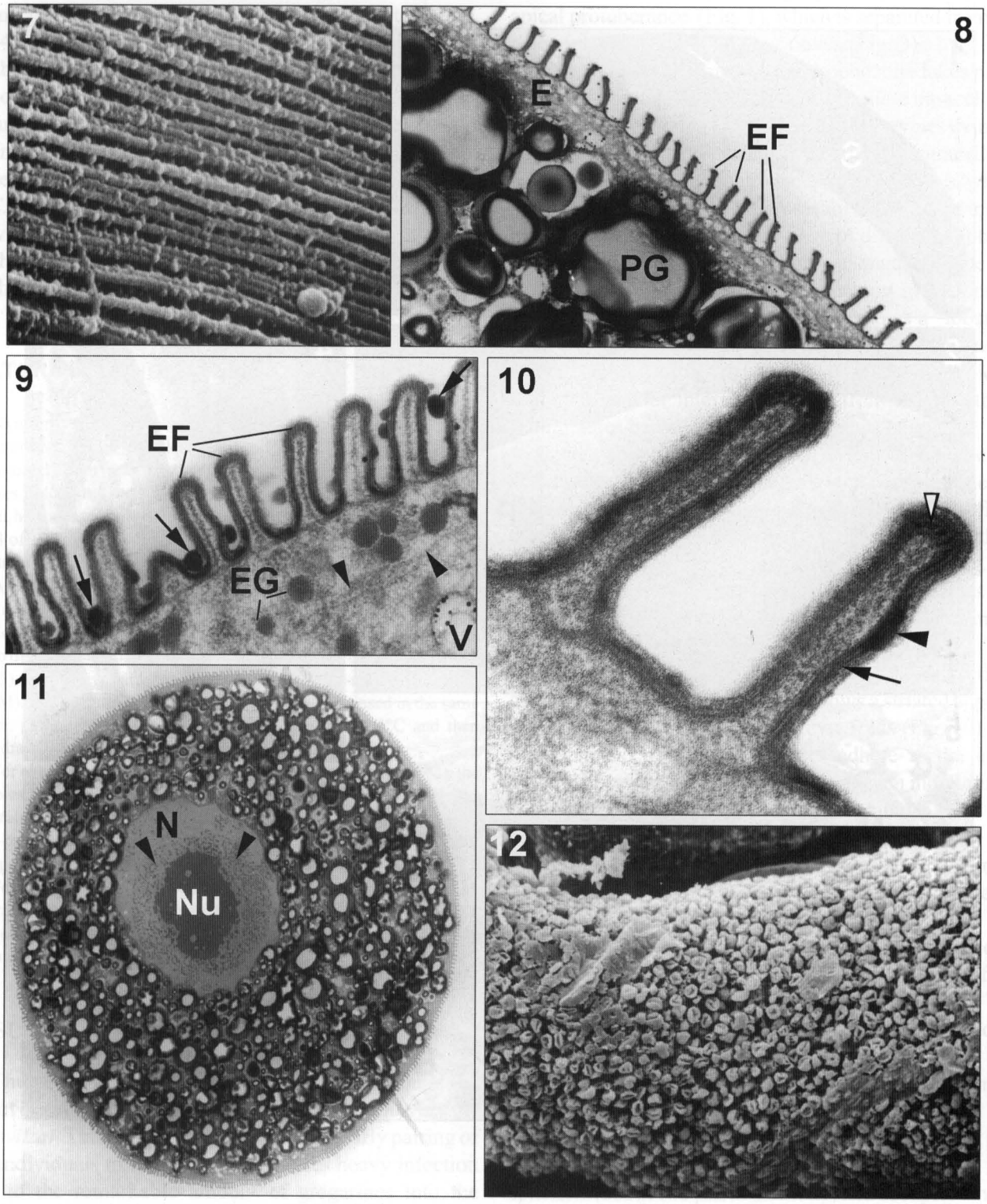
In the syzygy the entire surface of primite and satellite is delimited by an epicyte layer which forms a series of regular longitudinal folds (Fig. 7). Epicytes emerge from the thin peripheral ectocyte bounding the entocyte with its remarkable amount of paraglycogen granules (Fig. 8). Ectocyte includes 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).

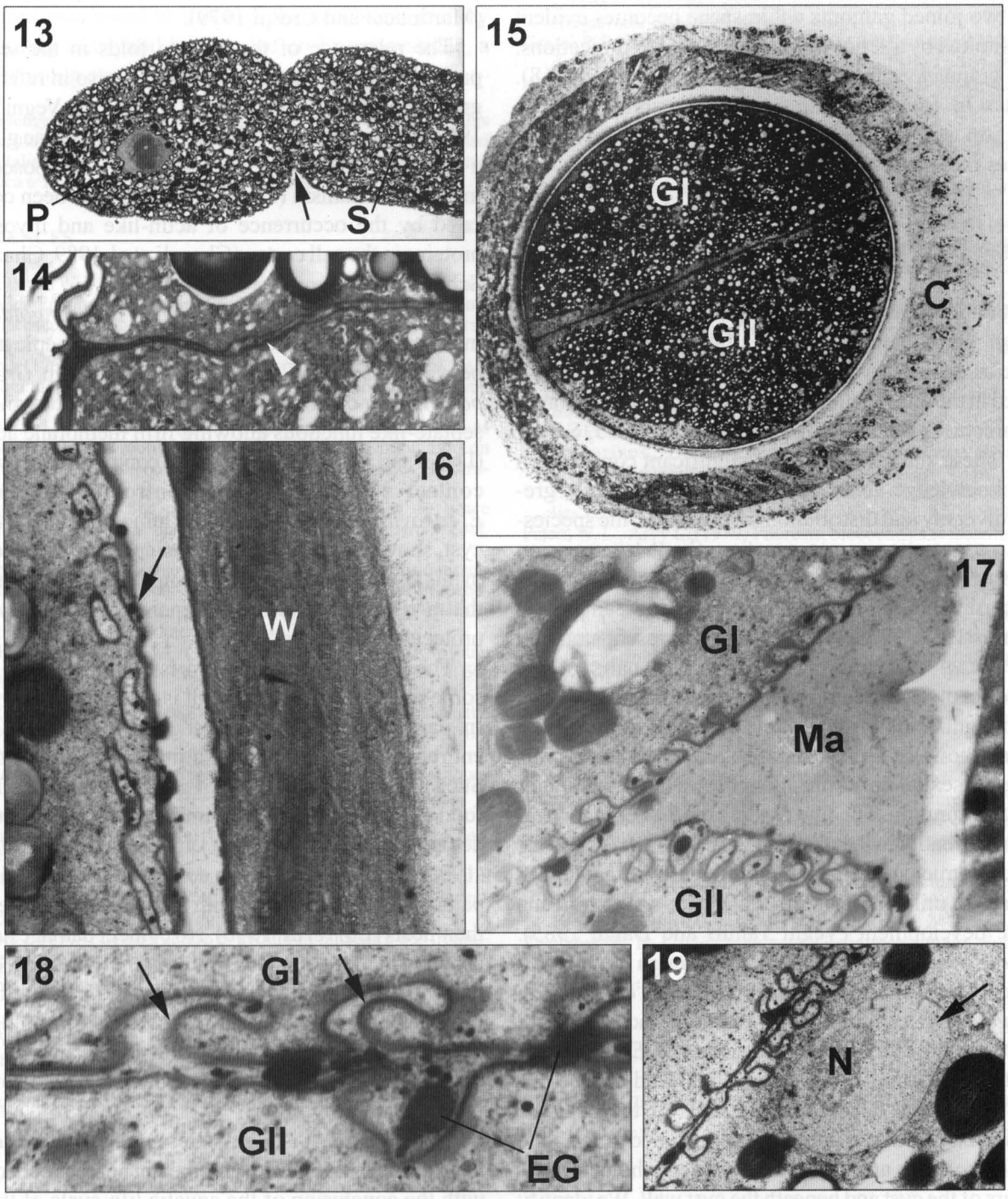
A single gametocyst has been found in the terminal part of the midgut (Fig. 15) of a specimen of *B. rhodani*. It is spherical (about 140 μm in diameter) and occupies almost the entire gut lumen. The gametocyst is uniformely enveloped by a multilayered wall of 1.8 μm in thickness (Fig. 16). A homogeneous 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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