# ULTRASTRUCTURAL STUDIES ON THE DEVELOPMENT OF THE GREGARINE *ENTEROCYSTIS RACOVITZAI* IN THE GUT OF *BAETIS RHODANI* (EPHEMEROPTERA, BAETIDAE)

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**ABSTRACT**

Different developmental phases have been observed in the gregarine *Enterocystis racovitzai* living in *Baetis rhodani* (Insecta: Ephemeroptera): the young gregarine in the midgut cell epithelium; the syzygy in the midgut lumen and the cyst in the terminal portion of the midgut. Gregarines in mature syzygy exhibit characters useful for their identification. Even though the growing phases are difficult to identify, we have tentatively assigned to this species the observed single gamonts and individuals in pairs. Among the ultrastructural features, epicyte modifications and the presence of gametes in formation are described. A synopsis on the occurrence of various species of *Enterocystis* gregarines associated with Ephemeroptera is also reported.

## INTRODUCTION

The genus *Enterocystis* was created by Zwetkow (1926) for *E. ensis*, a gregarine parasite of the larval *Caenis*. Since then, various species of gregarines attributed to *Enterocystis* have been collected from several species of Ephemeroptera (see reviews in Geus, 1969; Arvy and Peters, 1973; Peters and Arvy, 1979).

The representatives of the genus *Enterocystis* are characterised by the intracellular development of the young gregarine, the lack of a septum between proto- and deutomerite, and by an early pairing of the gamonts to form syzygy (Desportes, 1966). The lack of a real septum subdividing the gregarine cell has been also observed in *Gamocystis ephemerae*, a feature reported by Rühl (1976) as a valid trait to reconsider the taxonomic position of this species. Desportes (1963), on the basis of some similarities with other gregarines belonging to *Enterocystis*, proposed to attribute this species to this last genus. The doubtful allocation of *G. ephemerae* has been previously pointed out by Codreanu (1940).

So far, gregarines belonging to *Enterocystis* are exclusive to Ephemeroptera. They have been mainly collected from Baetidae, as reported by Desportes (1966), who sketched some

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distinct morphological features of the various parasitic species which are useful for specific attribution. Among Baetidae, larvae of *Baetis rhodani* constitute the preferential host for several gregarines, such as *E. ensis*, *E. fungoides* and *E. racovitzai* (Desportes, 1963; 1964; and see table I herein).

The ultrastructure of the syzygy of *Enterocystis* is known for *E. fungoides* (Desportes, 1974) and we have recently described some ultrastructural aspects of *E. racovitzai* (Gaino and Rebora, 1998).

The purpose of this study is to present additional data on the ultrastructure of *E. racovitzai* during its development.

## MATERIAL AND METHODS

Nymphs of *Baetis rhodani* (Pictet), which were collected in the Lemme stream (Voltaggio, Piedmont-Italy, from 18 October 1996 to 10 April 1998) contained gregarines belonging to *Enterocystis racovitzai* Codreanu, 1940. Among 48 examined insects, gregarine were found in 10 individuals. These gregarines were found both intracellularly and in syzygies in the alimentary canal. A single mayfly with dark wing-pads showed a mature gametocyst in the terminal tract of the midgut.

Gregarines removed from the midgut were observed *in vivo* under both light and interference contrast microscopes.

For ultrastructural investigation, selected material was differently fixed: (a) 1 hour in Karnovsky's medium (1965); (b) 1 hour in glutaraldehyde diluted to 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 hour at 4°C, and then dehydrated in a graded ethanol series.

For observations under the 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 515 at an accelerating voltage of 18 kv.

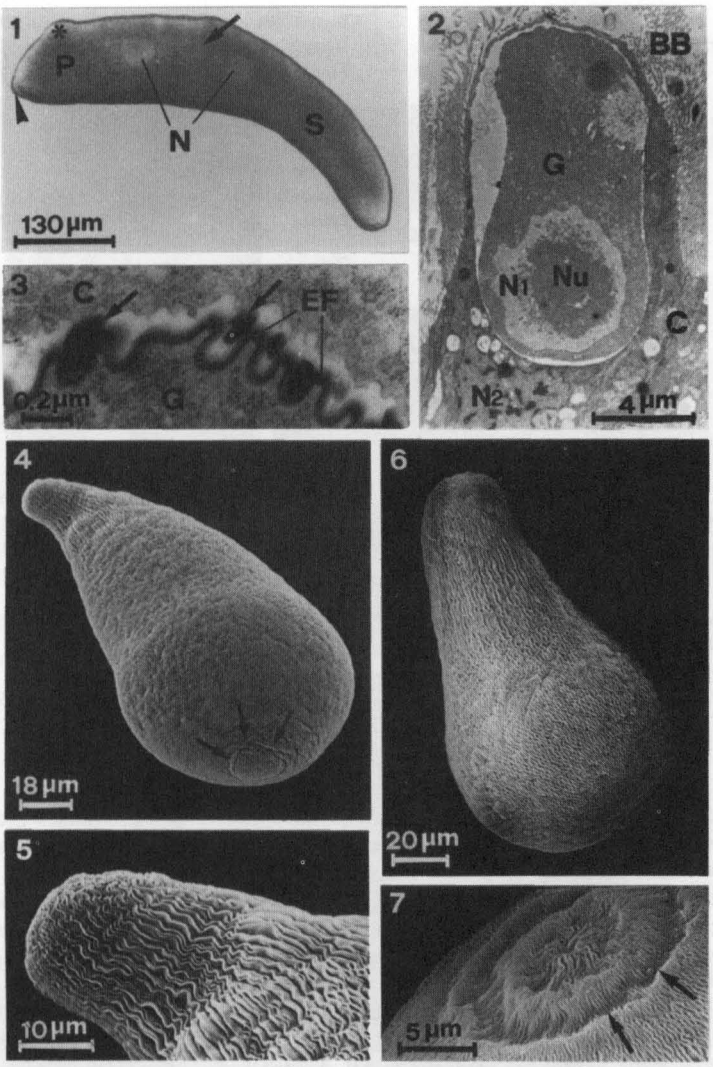
For observations under the transmission electron microscope (TEM), the material was embedded through propylene oxide in Epon-Araldite. Thin sections were obtained using a Reichert ultramicrotome, stained with uranyl acetate and lead citrate, and were examined under a Philips EM 400 T.

## RESULTS

Frantzius (1848) described the first gregarine, *Zygocystis ephemerae*, in the mayfly *Ephemera vulgata*. The taxonomic position of this gregarine has repeatedly changed as time went by (review in Arvy and Peters, 1973), because the species was then redescribed as *Gamocystis francisci* by Schneider (1882) and changed to *Gamocystis ephemerae* by Labbé (1899, in Codreanu, 1940), to *Enterocystis* (*Gamocystis*) *ephemerae* by Codreanu (1940) and back to *Gamocystis ephemerae* by Geus (1969). A new species was described by Geus (1969), *G. cloeonis*, a parasite of *Cloeon* sp.

A synopsis of the presence of *Enterocystis* gregarines in the gut lumen of mayflies from various sampling areas is reported in Table I. This synopsis does not include other gregarines whose attribution to *Enterocystis* is doubtful (such as *Gamocystis ephemerae*). It emerges that Baetidae during their aquatic life cycle are the main host for the parasites. On occasion, the presence of tentatively identified enterocystids has been reported in the larvae of *Ephemerella*, *Leptophlebia*, *Caenis* and *Siphlonurus* mayflies (Shtein, 1960).

The absence of a species-specific relationship between host and parasite makes a specific attribution difficult at the early stage of gregarine development in the gut lumen, where the individuals join together to form syzygy. Better identification is possible as the




**Figs. 1-7.** Different developmental phases of *Enterocystis racovitzai* from the midgut of *Baetis rhodani* under interference contrast microscope (1), TEM (2,3), SEM (4-7). 1- Mature syzygy. Note the nucleus (N) of the primite (P) and of the satellite (S), the septum (arrow), the apical protuberance (arrowhead) and the lateral lobe (asterisk); 2- Intracellular young gregarine (G) which protrudes towards the gut lumen. C= midgut cell with its brush border (BB); $N_1$= nucleus of the gregarine and nucleolus (Nu); $N_2$= nucleus of the midgut cell; 3- Epicyte folds (EF) of the intracellular young gregarine (G). Note the electron-dense material (arrows) among the epicyte folds. C= midgut cell; 4- Pear-shaped young gregarine with its slightly uplifted apical region (arrows); 5- Magnification of the "tail" of the pear-shaped young gregarine; 6- Pear-shaped gregarine in a more advanced phase of growth; 7- Detail of the apical protuberance of the pear-shaped gregarine. Note the circular deep groove (arrows).

**Table 1.** *Enterocystis* gregarines in the gut lumen of mayflies from various sampling areas.

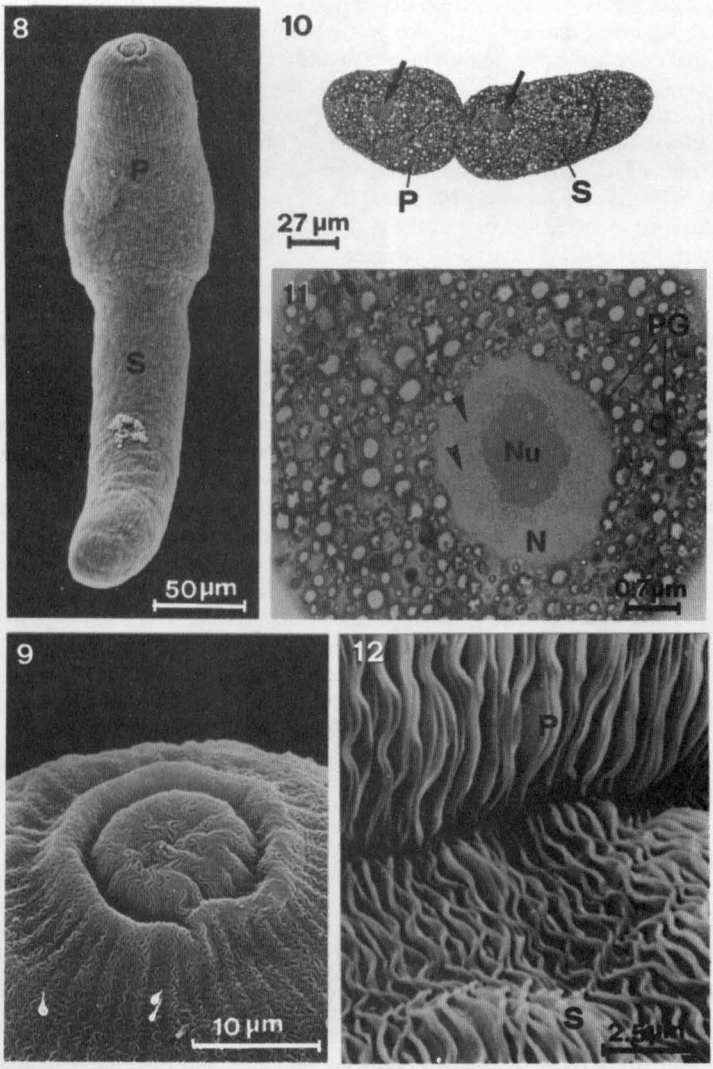
| Gregarine | Ephemeroptera | Locality | Author |
|---|---|---|---|
| *Enterocystis ensis* Zwetkow, 1926 | *Caenis sp.* | Russia (Peterhof) | Zwetkow, 1926 |
| | *Cloeon sp.* | Russia | Bobyleva, 1963 |
| | *Baetis rhodani* | France (East Pyrenees) | Desportes, 1964 |
| *E. fungoides* Codreanu, 1940 | *Baetis rhodani* | Rumania | Codreanu, 1940 |
| | *Baetis rhodani* | France | Desportes, 1963 |
| | *Baetis rhodani* | France (East Pyrenees) | Desportes, 1964 |
| | *Baetis vernus* | Rumania | Codreanu, 1940 |
| | Baetidae | France | Desportes, 1974 |
| *E. grassei* Desportes, 1963 | *Baetis vernus* | France | Desportes, 1963 |
| | *Baetis tenax* | France | Desportes, 1963 |
| | *Heptagenia flava* | France | Desportes, 1966 |
| | *Ecdyonurus sp.* | France | Desportes, 1966 |
| | *Epeorus torrentium* | France | Desportes, 1966 |
| *E. palmata* Codreanu, 1940 | *Baetis buceratus* | Rumania | Codreanu, 1940 |
| *E. racovitzai* Codreanu, 1940 | *Baetis vernus* | Rumania | Codreanu, 1940 |
| | *Baetis rhodani* | France | Desportes, 1963 |
| | *Baetis rhodani* | France (East Pyrenees) | Desportes, 1964 |
| | *Baetis rhodani* | Italy | Gaino & Rebora, 1998 |
| *E. rhithrogenae* Codreanu, 1940 | *Rhithrogena semicolorata* | Rumania | Codreanu, 1940 |
| *Enterocystis* sp. | *Baetis rhodani* | Rumania | Codreanu, 1940 |

maturation proceeds and the paired gregarines differentiate characteristics useful for a proper diagnosis. In this regard, the syzygy of *E. racovitzai* consists of a primite showing a lateral short extension, an apical protuberance, and a satellite (Fig. 1).

The onset of the gregarine development takes place inside the cells of the midgut. The first intracellular phase that can be identified as a gregarine under TEM is represented by an individual that shows a slightly elongated shape and is included into a vacuole (Fig. 2). The presence of the parasite deforms the apical part of the epithelial cell that markedly protrudes towards the gut lumen. The cytoplasm of the gregarine is fairly homogeneous and does not contain special inclusions, and the nucleus is located in the region opposite to the brush border of the host cell. Irregular epicyte folds are evident and electron-dense granules occur between them (Fig. 3).

The youngest gregarine found in the gut lumen before pairing is pear-shaped (Fig. 4). Under SEM its body surface reveals the differentiation of longitudinal epicyte folds that converge into a slightly uplifted apical region. The posterior region extends into a short "tail" along which the epicyte folds are looser than on the rest of the gregarine's surface (Fig. 5). Other pear-shaped gregarines in a more advanced phase of growth (Fig. 6) show modifications of both apical and posterior regions. These changes result in a better definition of the apical uplifted area, consisting of a central protuberance delimited by a deep groove (Fig. 7) and in the disappearance of the tail (Fig. 6).

Gamonts in pairs show a remarkable variability in morphology during the course of their growth in the gut lumen. The gregarines growing in pairs showed a clear dimorphism




**Figs. 8-12.** Syzygy of *Enterocystis racovitzai* from the midgut of *Baetis rhodani* under SEM (8,9,12) and TEM (10,11). 8- Syzygy. Note that the primite (P) is shorter than the satellite (S). 9- Detail of the apical uplifted area of the syzygy in the Fig. 8; 10- Syzygy in longitudinal section. The position of the nuclei is indicated by arrows. P= primite; S= satellite; 11- Section of a syzygy showing the nucleus (N) of the primite. Note the nucleolus (Nu) and the scattered filaments (arrowheads); PG= paraglycogen granules; 12- Contact region between primite (P) and satellite (S) showing the different arrangement of the epicyte folds in the early syzygy.

with a primite shorter than the satellite (Fig. 8). Typically, the satellite tends to narrow as the gregarine maturation proceeds (Fig. 8). Concomitantly, the apical uplifted area acquires a sucker-like appearance (Fig. 9).

The position of the nuclei in the primite and in the satellite is clearly visible under TEM in a longitudinal section (Fig. 10). The nucleus of each individual shows an irregular border, and contains a nucleolus with an electron-dense region and scattered filaments (Fig. 11).

In young gregarines, dimorphism is also indicated by a different arrangement of the epicyte folds: looser and taller in the primite than in the satellite (Fig. 12). This feature tends to disappear during syzygy maturation.

Epicyte folds emerge from the thin peripheral ectocyte that bounds the endocyte (Fig. 13). The ectocyte includes vacuoles, electron-dense inclusions and fibrillar components (Fig. 14). The paraglycogen granules, which represent typical storage material, accumulate in the endocyte (Fig. 13).

In cross-sections, the epicytes are delimited by a multilayered border that presents a striated appearance in the apical part of the epicyte folds (Fig. 15). Electron-dense granules are occasionally found adherent to the cytomembranes (Fig. 15) and are usually interposed between consecutive folds (Fig. 14).

In pairing gamonts, observed under SEM, the epicytes are modified along their contact region to form a narrow band (Fig. 16). Longitudinal sections of the contact area confirm the drastic change in the epicyte fold pattern: the cell membranes of the associated individuals run in parallel fashion showing a close adhesion (Fig. 17).

A single gametocyst has been found in the terminal part of the midgut of a mature nymph of *Baetis rhodani*, which was about to emerge. The cyst has a spherical shape (about 140 μm in diameter) (Fig. 18) and almost completely occupies the gut lumen (Figs. 18, 19). The cyst is delimited by a wall (1.8 μm in thickness) (Fig. 20) with a multilayered organization (Fig. 21). Below the envelope the surface of the gamonts appears remarkably modified. Indeed, the epicyte folds drastically reduce in length up to their final disappearance (Fig. 20). In contrast, modified epicyte folds appear along the contact area, thus making more evident the boundary between the pairs (Fig. 22). Mucous material accumulates in the space leading to the contact area between the pairing gamonts. Spherical cells of 2.5-3 μm, characterised by a homogeneous cytoplasm and a wide nucleus, appear among the abundant paraglycogen granules that tend to shadow these minute cells. Their morphology is consistent with their identification as developing gametes (Fig. 23).

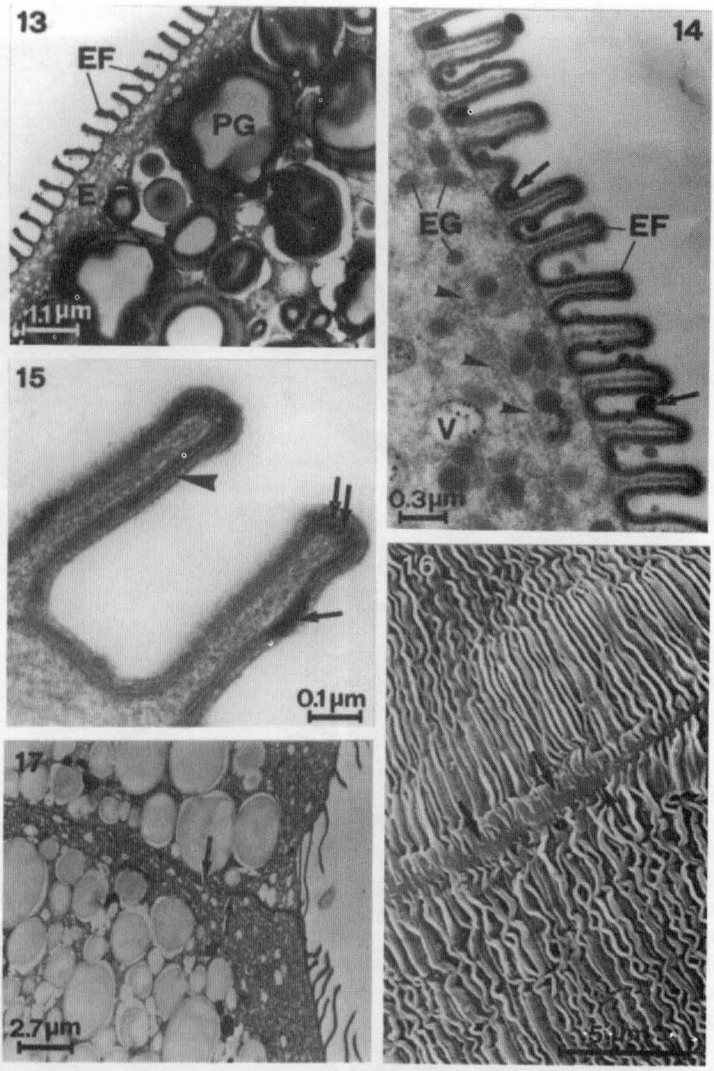
No further developmental steps, or other cyst formation, have been observed in spite of our efforts.

## DISCUSSION

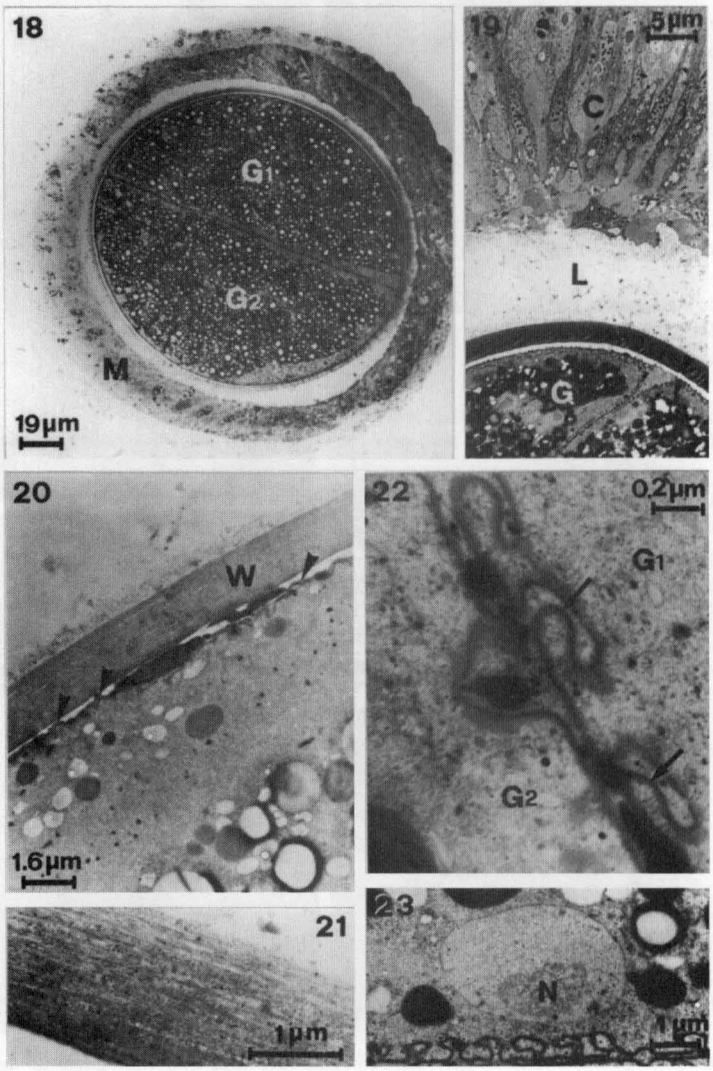
The main feature distinguishing Gregarinidae from Enterocystidae, the latter which includes only the genus *Enterocystis*, is the lack of a septum between proto- and deutomerite in enterocystids. Therefore, it is quite surprising that *Gamocystis*, in spite of its lack of septate gregarines (Rühl, 1976), is still included in Gregarinidae, thus making the lack of a septum not an exclusive feature of the genus *Enterocystis*.

The present study on *E. racovitzai* confirms previous observations (Codreanu, 1940; Desportes, 1963; Geus, 1969) that paired gamonts constitute the most common phase of development of this parasite. Indeed, intracellular gregarines and single individuals can be observed only rarely. The joined partners grow together and, except for the epicyte folds, their sexual dimorphism increases with maturation, thereby emphasizing the morphological differences between primite and satellite. Primite undergoes a major diversification consisting of lateral expansions and an apical protrusion. Our SEM images showed that this apical protrusion is already present in single individuals living in the midgut lumen and could represent a marker useful for identifying this gamont.

The fine organization of *E. racovitzai* conforms broadly to that described for other gregarines regarding the epicyte structure and the remarkable amount of paraglycogen




**Figs. 13-17.** Details of the mature syzygy of *Enterocystis racovitzai* from the midgut of *Baetis rhodani* under TEM (13-15,17) and SEM (16). 13- Sequence of epicyte folds (EF) emerging from the ectocyte (E) that bounds the endocyte-rich area of paraglycogen granules (PG); 14- Accumulation of extracellular electron-dense material (arrows) between epicyte folds (EF) emerging from the endocyte including vacuoles (V), electron-dense granules (EG) and fibrillar components (arrowheads); 15- Epicyte folds delimited by a multilayered border (arrowhead) with a striated appearance (arrows). Note the electron-dense granules adherent to the cytomembranes (arrow); 16- Contact region in the mature syzygy. Note the narrow band (arrows) between the two gamonts. 17- Longitudinal sections of the contact area showing the close adhesion (arrows) between the two gamonts.




**Figs 18-23.** Gametocyst of *Enterocystis racovitzai* in the midgut of *Baetis rhodani* under TEM. 18- Gametocyst in the midgut (M) showing gamonts in association (G1-G2); 19- Epithelial cells (C) facing the gametocyst (G). L= midgut lumen; 20- Gametocyst wall (W). Note the remarkably reduced epicyte folds (arrowheads); 21- Detail of the gametocyst wall showing its multilayered organization; 22- Modified epicyte folds (arrows) along the contact area between gamonts (G1-G2); 23- A gamete in formation. N= nucleus.

granules necessary for parasite development (Vegni Talluri and Dallai, 1985). Another common feature is represented by the extrusion of electron-dense material through the epicytes, a feature already shown by Desportes (1974) in *E. fungoides*. On the basis of the resemblance of our images with those of authors who used histochemical techniques, we believe that this material is composed of mucopolysaccharides. As stressed for other gregarines, this material is associated with the life cycle of the parasite, and it is important for gliding movements (King *et al.*, 1982; Dallai and Vegni Talluri, 1983; Vegni Talluri and Dallai, 1983, Ghazali *et al.* 1989; Ghazali and Schrével, 1993), cyst wall differentiation and, presumably, the homogeneous matrix we observed between the gamonts inside the cyst.

SEM images of young syzygy showed that epicyte folds of the primite are slightly longer than those of the satellite, thus emphasizing that ectocyte organization could be the expression of an early sexual dimorphism. As observed by Desportes (1974), the epicyte folds disappear along the contact zone and the opposite membranes tend to run in a parallel fashion. In *E. racovitzai,* they are more closely associated, a feature consistent with the occurrence of a specialization strengthening the joining of partners. Indeed, the representatives of Enterocystidae are peculiar among gregarines for their early pairing, a feature that needs a stable connection between the partners during their further growth. The mechanical role of cell junction in gamont association has been stressed by Dallai and Vegni Talluri (1988), who described the presence of a septate junction in the syzygy of *Gregarina polymorpha.*

A certain level of plasticity in the shape of the epicyte folds along the contact region is suggested by the comparison between the syzygy not included in the cyst and the syzygy wrapped in the cyst wall. Indeed, in the latter, the folds, even though reduced in length, become evident again, thereby making the joining between the partners more evident. This feature contrasts remarkably with the rest of the ectocyte surface where the folds are no longer visible.

We believe that the modifications of the epicytes reflect their function in the life cycle of the parasite, while the gamonts are growing in the gut lumen or are included in the cyst. The need for firm adhesion is mainly related to the gliding movement that is accomplished by vertical undulation of the epicyte folds (Walker *et al.*, 1979; MacKenzie and Walker, 1983), which ceases at the level of the contact region to avoid distortions (Dallai and Vegni Talluri, 1988).

According to previous observations (Desportes, 1963; Codreanu and Codreanu-Balcescu, 1979), cyst differentiation takes place at the end of the aquatic portion of the mayfly life cycle, in such a way that the cyst, with gametes in formation, is released before the emergence of the insect. This represents a successful strategy allowing the parasite to infect other individuals.

The attribution of the gregarines dissected from *Baetis rhodani* to *E. racovitzai* is mainly based on the comparison between the drawings reported in the literature for this species and for other gregarines that are parasites of Ephemeroptera (see references in Table 1). The mature syzygy offers a good opportunity for specific attribution, while the growing phases are difficult to determine. In this report, the latter are referred to *E. racovitzai* in consideration of the fact that so far only this gregarine has been found in the gut lumen of our specimens of *B. rhodani.*

In conclusion, our finding of *E. racovitzai* in Italian representatives of *B. rhodani* allowed us to identify some ultrastructural morphological traits that are useful for taxonomic purposes and to increase the knowledge on the interaction between host and parasite.

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