Acta Protozool. (2000) 39: 225 - 231

ACTA
PROTOZOOLOGICA

Ophryoglena sp. (Ciliata: Oligohymenophora) in *Caenis luctuosa* (Ephemeroptera: Caenidae)

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Summary. Sampling of mayfly nymphs belonging to *Caenis luctuosa* (Ephemeroptera, Caenidae) revealed that 5% were infected by an enormous number of ciliates of the genus *Ophryoglena*. Free moving ciliates were recognisable by observing the host animals *in vivo* under a stereomicroscope. The ciliates lived in the hemolymph and penetrated the wing pads and trochanteral junctions of the legs. After their removal from the host body, some specimens were reared in Petri dishes. *Ophryoglena* sp. formed cysts and failed to survive more than two days. In order to test the effect of the parasites on the host tissues, the ovarioles of some healthy and parasitised specimens were examined under TEM. Parasitic castration depends upon an early degeneration of the follicle cells, which were unable to envelope the egg within a firm epithelium. Eggs were blocked in their early phase of maturation since the integrity and activity of the follicular epithelium is essential for the ensuing synthesis of the egg envelopes (vitelline and chorionic layers). The low rate of parasitized mayflies hampers a full understanding of the life cycle of this ciliate and of its modality of spreading.

Key words: Ciliate, endoparasite, Ephemeroptera, *Ophryoglena*, SEM, TEM, ultrastructure.

INTRODUCTION

The first record of a ciliate parasitising insect hemolymph dates to Lichtenstein (1921), who described *Ophryoglena collini* in the nymphal hemocoel of the mayfly *Baetis* sp. living in a stream close to Montpellier (France). This ciliate is hematophagous and more generally histophagous, since it destroys gonads, muscles and the fat body of the host insects (Arvy and Peters 1973). Codreanu (1930) found ciliates belonging to *Ophryoglena* in the nymphs of *Rhithrogena* sp. and *Baetis* sp. from the Southern Carpathians (Rumania), but identified *O. collini* only in *Baetis* sp. Afterwards, Codreanu (1934) reported the occurrence of *Ophryoglena* in the nymphs of *Oligoneuriella rhenana* (cited as *Oligoneuria rhenana*) from the Gresse stream (a tributary of the Drac River before it flows into the Isere River). In 1972, Codreanu found specimens of *Ophryoglena* living in *Rhithrogena semicolorata* and *Oligoneuriella rhenana* and named a new species *O. ovariovora*. These ciliate species were differentiated because *O. collini* does not form cysts in the hemocoel of the host before its intraovaric multiplication, whereas, *O. ovariovora* develops cysts before ovarian

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invasion (Codreanu 1934, Codreanu and Codreanu-Balcescu 1979). In addition, Codreanu (1930, 1934) stressed that the life cycle of the parasite differs in male and female mayflies. Only in females does the parasite enter the gonads to feed on egg inclusions with resulting destruction of the ovaries (the coeloconic phases which may occur within or without cysts according to species and host). The imaginal females lay *Ophryoglena* instead of eggs, so that ciliates have good opportunities to infect other mayfly nymphs (Codreanu 1934).

Even though the developmental cycle of both species of *Ophryoglena* is unclear, the above literature stressed that parasite-induced host castration is due to invasion of the ovary by ciliates. These appear as free moving coelomic forms or as individuals emerged from cysts that acquire their final stage within the ovaries.

In our samples of nymphs of *Caenis luctuosa*, we found a few instances of *Ophryoglena* sp. parasites. The low rate of parasitism together with the presence of only some developmental phases of the ciliate hampered a specific attribution to be made. The parasitic structure and its interaction with the mayfly were studied at the ultrastructural level. These observations include a new host genus and show that parasitic castration is not due to direct aggression of the ciliate to the ovary.

MATERIALS AND METHODS

Female nymphs of *Caenis luctuosa* (Burmeister 1839) (Caenidae) were collected in the Tescio Stream (Bastia Umbra, Perugia, Italy) at the end of July 1999. A few individuals showed parasites under a stereomicroscope. A survey carried out on specimens of *Caenis luctuosa* sampled in several Umbrian Streams and of *Caenis horaria* from Lake Piediluco revealed that parasitized specimens were limited to the Tescio Stream. Anaesthetised mayflies (1% chloral hydrate) with or without parasites were dissected in order to remove the parasites belonging to *Ophryoglena* sp. (Ciliata, Oligohymenophora, Hymenostomata, Histophaga) from the hemocoel. In addition, ovarioles of both mayfly groups were extracted from the abdomen. Selected samples were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 1 h at 4°C.

For SEM analysis, samples were dehydrated in graded ethanol series, critical point dried using a CO_2 Pabisch CPD apparatus, mounted on stubs with silver conducting paint, coated with gold palladium in a Balzers Union Evaporator, and observed with a Philips EM 515 SEM. For TEM analysis, material was postfixed in osmium tetroxide (1% in phosphate buffer) for 1 h at 4°C, repeatedly rinsed in the same buffer, dehydrated in graded ethanol series, and embedded in Epon-Araldite mixture resin. Thin sections, cut on a Reichert ultramicrotome, were collected on formvar-coated copper grids, stained with uranyl acetate and lead citrate, and observed with a Philips EM 208 TEM.

Information on the movements of the ciliates towards their host and their survival out of it was acquired by observing the behaviour of the specimens *in vivo*.

RESULTS

Of sixty specimens of *Caenis luctuosa* observed under a stereomicroscope, only three were infected by *Ophryoglena* sp. These ciliates lived in the mayfly hemolymph and their presence was already detectable by observing the host from its dorsal side, even though they were partially shaded by the pigments of the wing pads (Fig. 1), and abdominal segments. From the ventral side, the accumulation of ciliates in the body was evident through the unpigmented sterna (Fig. 2). The astonishing number of ciliates (about three hundreds per individual) filled up the hemolymph to the coxa since even in this region SEM images showed these organisms in clumps of two/three individuals (Fig. 3).

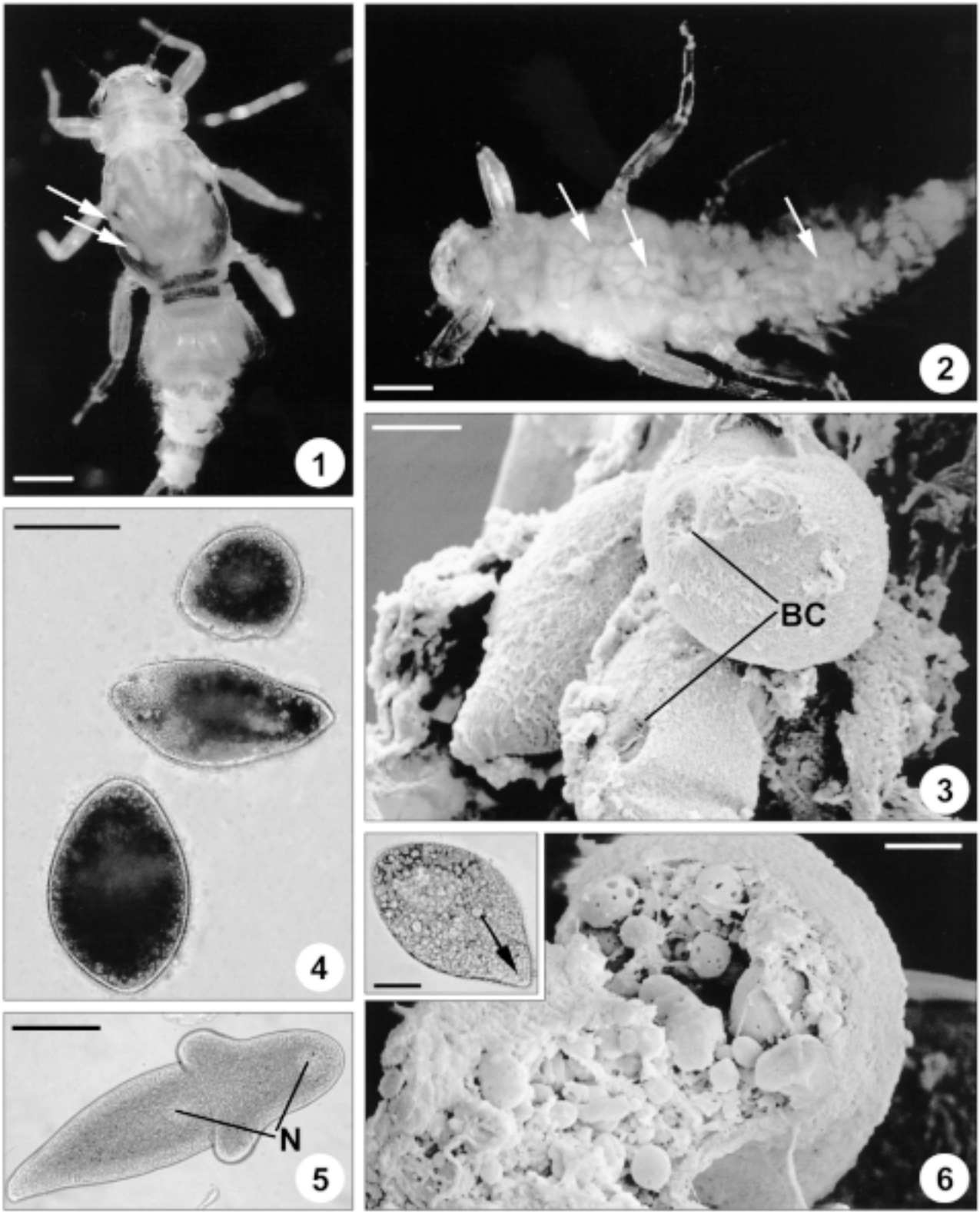
After their removal from the hemolymph, about one hundred ciliates were cultured in Petri dishes using the same stream water where infected specimens of *C. luctuosa* had been collected. In this medium, ciliates were freely moving and showed a different appearance, which probably reflected different stages of their complex life cycle (Fig. 4).

In order to ascertain the possible orientation of parasites towards the host, a young nymph was added to the Petri dish containing freely moving ciliates. No oriented movement of ophryoglenas was detected.

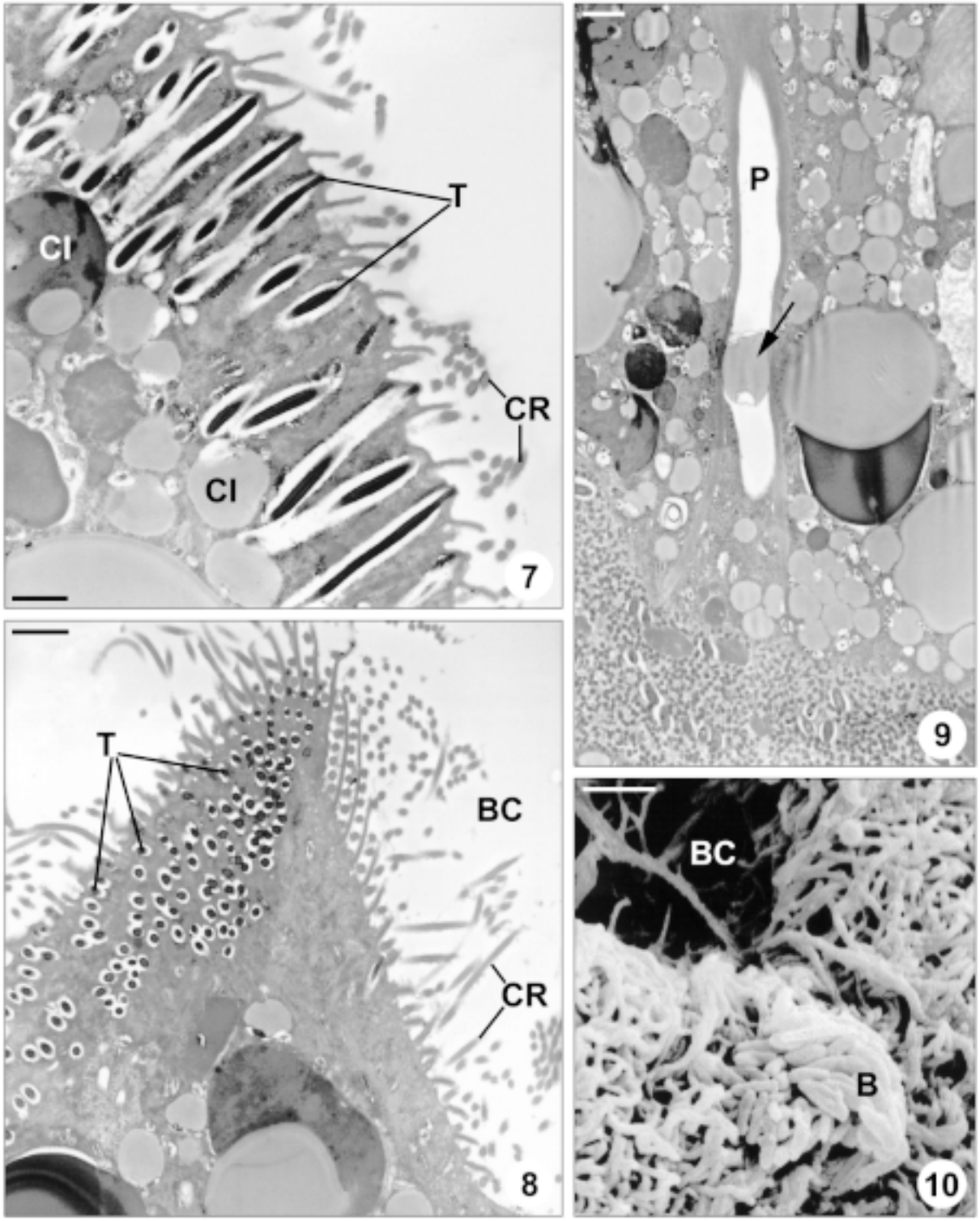
Two hours after removal from the host hemolymph, most of the parasites (about 60%) lost mobility and formed cysts. Twenty-four hours after the beginning of our experiments, bacterial attack affected both cysts and uncysted ciliates causing death of all the reared organisms.

By dissecting a specimen of *C. luctuosa* hosting *Ophryoglena* sp. into the hemolymph, some gregarines whose paired individuals formed syzygies were also removed from the gut. Their morphology was consistent with the species *Enterocystis fungoides* (Fig. 5). This species was originally described by Codreanu (1940) in other mayflies belonging to Baetidae and has been studied more recently by Desportes (1974) under TEM.

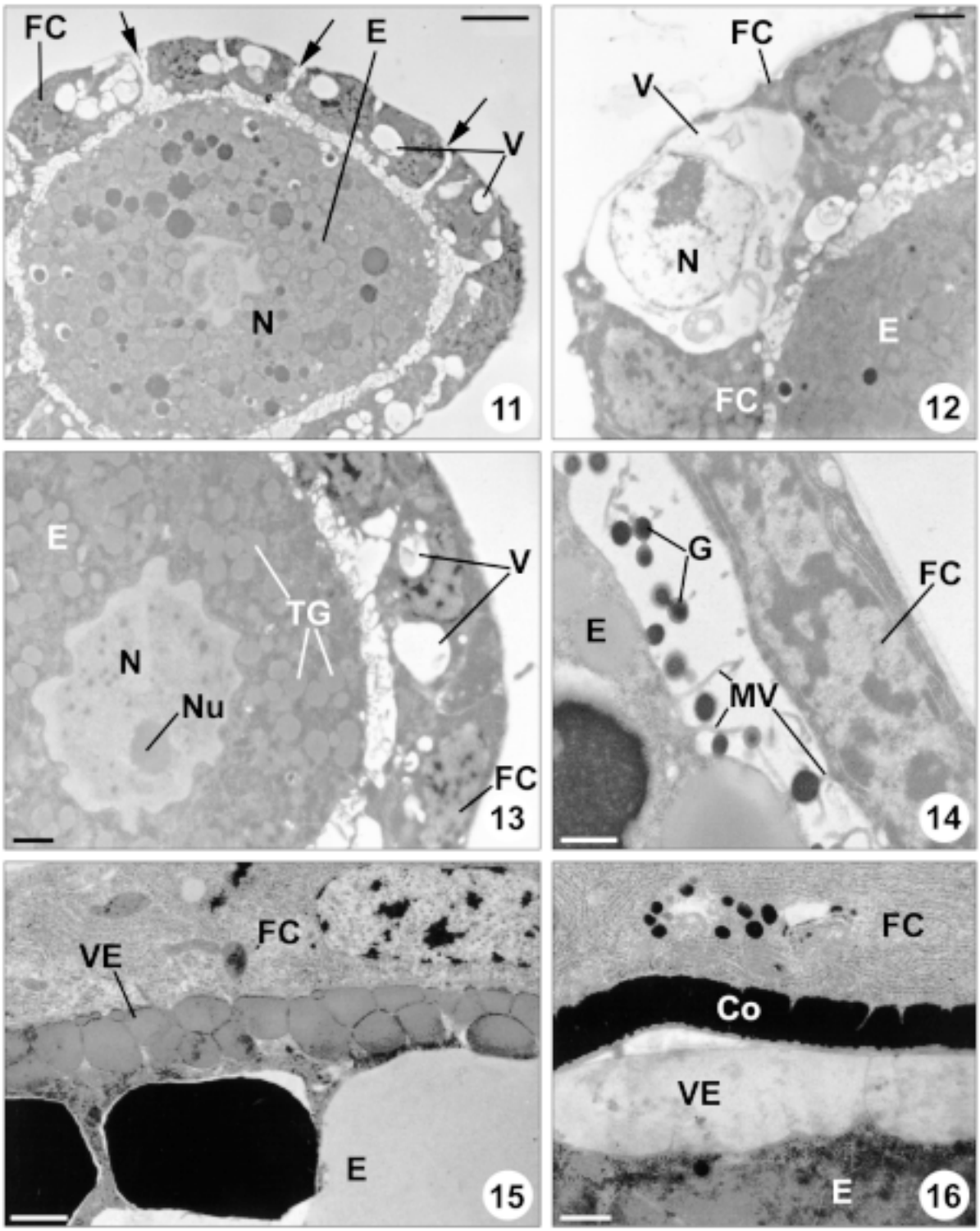
Among the freely moving *Ophryoglena* sp. removed from the hemolymph, trophonts were easily recognisable because of their spatulate apical region (inset of Fig. 6). Their cytoplasm was filled with inclusions of various sizes located just beneath the cell surface, as proved by



Figs. 1-6. Dorsal view of a parasitized nymph of *Caenis luctuosa*. Note the ciliates (arrows) under the unpigmented wing pads; **2 -** ventral view of a parasitized nymph of *Caenis luctuosa*. Note the large amount of ciliates in its hemolymph (arrows); **3** - SEM micrograph of three specimens of *Ophryoglena* in the joint between thorax and legs of *C. luctuosa*, **4 -** freely moving ciliates whose morphology reflects different stages of their life cycle; **5 -** the gregarine *Enterocystis fungoides* from the gut of a mayfly specimen parasitized by ciliates; **6 -** a broken ciliate under SEM showing its inclusion-laden cytoplasm. The inset shows a trophont of *Ophryoglena* with its spatulate apical region (arrow). BC - buccal cavity, N - nuclei. Scale bars - **1** - 500; **2** - 400; **3** - 25; **4, 5** - 50; **6** - 10; frame - 25 µm



Figs. 7-10. TEM view of the peripheral cell border of the trophont of *Ophryoglena* sp. including trichocysts; **8** - region of the buccal cavity lacking trichocysts; **9** - pharynx showing the flow of amorphous material (arrow); **10** - a clump of bacteria adherent to the cilia of the buccal cavity. B - bacteria, BC - buccal cavity, CI - cytoplasmic inclusions, CR - ciliary rows, P - pharynx, T - trichocysts. Scale bars - **7** - 1; **8-10** - 2 µm



Figs. 11-16. Transversal section of the follicle cells of a parasitized specimen of *C. luctuosa* showing the egg blocked at an early phase of vitellogenesis. Note the loose juxtaposition (arrows) between follicle cells, and their vacuoles; **12** - detail of the follicle epithelium showing marked vacuolisation around the nucleus of a follicle cell; **13** - a region of the egg filled with electron-translucent granules, which shows well-preserved nucleus and nucleolus. Note the vacuoles inside the superimposed follicle cells; **14** - ovarian follicle of a non-parasitized specimen. Precursor material of the vitelline envelope is evident as electron-dense granules. These are accumulated in the region crossed by microvilli emerging from both egg and follicle cell; **15** - in non-parasitized specimens the vitelline envelope is interposed between follicle cells and egg; **16** - late phase of egg maturation in a non-parasitized specimen showing the vitelline envelope and the chorion around the egg. Co - chorion, E - egg, FC - follicle cell, G - electron-dense granules, MV - microvilli, N - nucleus, Nu - nucleolus, TG - electron-translucent granules, V - vacuoles, VE - vitelline envelope. Scale bars - **11** - 3; **12,13** - 1; **14** - 0.5; **15** - 0.8; **16** - 0.1 µm

experimentally broken cells observed under SEM (Fig. 6). TEM investigation showed that there is a clear difference between the region with cell inclusions and the peripheral cell border of the trophont. This included a series of trichocysts of high electron density, arranged in parallel fashion (Fig. 7) along the cell perimeter, except for the buccal cavity where only ciliary rows persisted (Fig. 8). Longitudinal sections of the pharynx showed the flow of amorphous material (Fig. 9). Bacteria were occasionally seen adhering to the cilia of the buccal cavity (Fig. 10).

Ultrathin sections of the ovarioles removed from healthy specimens used as control and from a nymph hosting *Ophryoglena* sp. revealed that the ensuing process of egg envelope differentiation remarkably reflected the presence of the parasite. In the follicles of the parasitized mayfly, follicle cells enveloping each egg, blocked in an early phase of vitellogenesis, were loosely juxtaposed (Fig. 11). In addition, follicle cells showed marked vacuolisation of the cytoplasm, sometimes remarkably evident around the nucleus (Fig. 12), a finding indicating cell degeneration. In contrast, eggs did not show damage with both nucleus and cytoplasm well preserved (Fig. 13). In the control samples, follicles were in various phases of maturation and the follicle epithelium was formed by tightly adherent and elongated cells. Precursor material in the form of electron dense granules accumulated in the region between the egg and the follicle epithelium, thereby constituting an infant form of the vitelline envelope (Fig. 14). This region was crossed by microvilli protruding from both the egg surface and apical follicle cell border (Fig. 14). In the control samples, the egg layer formation proceeded giving rise to the vitelline envelope (Fig. 15) and to the superimposed chorion (Fig. 16).

DISCUSSION

The presence of parasites in mayfly nymphs has been widely illustrated (Arvy and Peters 1973, Codreanu and Codreanu-Balcescu 1979, Hominick and Welch 1980, Tokeshi 1988, Jacobsen 1995, Gonser and Spies 1997, Vance and Peckarsky 1997). The occurrence of endoparasitic Protozoa in mayflies is mainly related to gregarines hosted by various species (Gaino and Rebora 1998, Gaino and Rebora in press). The finding of *E. fungoides* in *Caenis luctuosa* increases the number of mayfly genera parasitised by this protozoan, so far limited to Baetidae (Gaino and Rebora in press). The only endoparasitic ciliates associated with mayfly nymphs are two species of *Ophryoglena* (*O. collini* and *O. ovariovora*) having the same "ovarian phase" (Canella and Rocchi-Canella 1976).

The feeding activity of *Ophryoglena* sp. on the host tissue is evident in the flow of material through its pharynx and in the accumulation of inclusions in its cytoplasm.

Codreanu's observations stressed that males and females are differently involved in parasite transmission. In the former, the ciliates do not invade the gonads and die with their host after the mating flight. In the latter, the parasite destroys the ovaries and is laid in water through the oviduct. Since we have never observed an intra-ovarian phase of the parasite in *C. luctuosa*, we hypothesise that the protozoan may act as a histophagous parasite to spoil the host tissues and reserves without feeding directly on the ovarioles. By comparing nymphs of similar developmental phases, it was evident that the ovarioles of the parasitized mayfly were blocked at an early phase of egg differentiation, whereas, those of the control of the non-parasitized specimens showed follicles arranged in a gradual series of maturation. As a result, parasitized hosts exhibited a thin linear sequence of small undifferentiated follicles.

The ovary of Ephemeroptera consists of numerous meroistic telotrophic ovarioles (Gottanka and Büning 1993, Büning 1994) typically arranged side by side to form parallel rows. Each ovariole is delimited by a follicular epithelium. As for other insect groups, this is highly specialised for synthesis of precursor material, which is released for egg envelope organisation (Mathew and Rai 1975, Norton and Vinson 1982). Ultrastructural analysis of the ovarioles of the mayfly *Habrophlebia eldae* Jacob & Sartori, 1984 proved that the organisation and activity of the follicular epithelium changed according to the secretory function performed during oogenesis (Gaino and Mazzini 1990). In particular, this study illustrated that in the previtellogenic ovarian follicles, the follicle cells are packed in a columnar epithelium with cells interconnected by gap junctional plaques and tightly interlocked with the egg surface through microvilli. The most outstanding features of the parasitized *Caenis* were (a) the lack of a firm connection between cells, which is essential for the creation of a thin epithelium around the growing egg, and (b) the relevant number of empty vacuoles inside the follicle cell cytoplasm. These features are consistent with follicle cell degeneration that could result from the astonishing growth and development of this ciliate in the hemolymph of the host. As

a consequence, ciliate parasites interfere with the normal development of the female gonads of the host and with host reproduction. This parasitic castration constitutes the most relevant consequence of ciliate invasion, although the life cycle of these protozoans in Ephemeroptera is still unknown. No male/female comparison could be carried out because no parasitised males were recorded in our samplings.

Codreanu (1934) noticed that 8% of the specimens of *Oligoneuriella rhenana* were infested by *Ophryoglena*. We have found a similar rate of infected animals (5%) in *C. luctuosa*.

The occurrence of bacteria close to the buccal cavity of some *Ophryoglena* sp., after their removal from the hemocoel of a specimen dissected in phosphate buffer, indicates that the parasitic activity facilitates the bacterial infection of the host hemolymph.

The low percentage of parasitized specimens hampers studies on the exploitation of the host tissues and on the invasion of the parasite during its dispersion phase.

Acknowledgements. This study was supported by a "Progetto d'Ateneo" grant of the University of Perugia.

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Received on 2nd February, 2000; accepted on 30th March, 2000