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ACTA PROTOZOOLOGICA
Vol. 28, No. 1, pp. 1–10 (1989)

Dynamics of the Submembrane Contractile System in Caffeine-derived Protoplasmic Droplets of *Physarum polycephalum* [1]

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Synopsis. The protoplasm-droplets freshly generated from plasmodial veins by caffeine treatment are rounded forms with reduced motile activity. Such forms, with the cytoplasm separated into hyalo- and granuloplasm, manifest spontaneous detaching of optically dense peripheral hyaloplasm layers from the plasma membrane and their shifting toward the granuloplasmic core. This phenomenon is regularly repeated every 6–15 s. The separation of the cortical sheets from the membrane is strongly promoted by some local anaesthetics which affect the membrane properties and membrane-cytoskeleton links, especially by 3% ethanol. In the caffeine-produced plasmodial droplets various forms of pinocytosis may be provoked by standard cationic inducers, as KCl and NaCl. Both observed phenomena may be a witness to high dynamic activity of the filamentous layer associated to the cell membrane of plasmodial droplets produced by the caffeine, in spite of their momentary incapability of migrating.

The dynamics of the contacts between the plasma membrane and the contractile filamentous layer leaning its cytoplasmic surface is crucial for the mechanism of amoeboid movement and endocytosis. The separation of the successive cortical layers from the plasma membrane and the reconstruction of the contractile apparatus beneath plasmalemma was recently described by Grębecki and Kwiatkowska (1988) in the hyalospheres, which are spherical forms of *Amoeba proteus* obtained by a heat shock, and characterized by the separation of

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hyaloplasm (around the periphery) and granuloplasm (in the centre). The dynamics of membrane-cortex interactions in this cell model can be seen in spite of the momentary loss of ability to locomote. Detaching of the new contractile layers from the plasmalemma is a spontaneous phenomenon, though it may be also provoked and intensified by some organic solvents and local anaesthetics, influencing the properties of the plasma membrane and its interactions with the cytoskeleton. The similar or possibly the same phenomenon was described in the slime moulds microplasmodia (Kuroda 1979, Kukulies et al. 1984) and in the fibroblasts (Heath and Dunn 1978, Sorrano and Bell 1982, Heath 1983).

It may be expected therefore that the separation of the contractile peripheral sheets of cytoplasm from the plasma membrane is a phenomenon occurring not only in amoeba hyalospheres, but may be manifested by other cell systems characterized by the presence of microfilamentous layer beneath the plasma membrane. Plasmodium of the acellular slime mould *Physarum polycephalum* is one of the best investigated examples of such systems. The plasmodium of *Physarum* is too large to be easily controlled under the microscope. Small plasmodial fragments, microplasmodia, obtained from the mature plasmodium are therefore often used for experiments. One of the better ways to obtain them is the caffeine method (Hatano and Oosawa 1971, Matthews 1977, Achenbach et al. 1979, Kukulies et al. 1983). A brief incubation of plasmodial fragments in 5-10 mM solutions of caffeine results in the formation of numerous spherical microplasmodia, called "caffeine droplets". Many caffeine droplets present the form morphologically identical to the hyalospheres of *Amoeba proteus*: stratification of the contents into a wide hyaloplasmic ring and central granuloplasmic core. All caffeine-induced protoplasmic droplets are membrane covered. They have a reduced motile activity (no locomotion), but the granular protoplasm inside them may display rhythmic or continuous motion (Matthews 1977, Kuroda 1979). The incapability of locomoting is caused by a characteristic caffeine effect, which delays the development of the plasmalemma invagination system, necessary for migration of a microplasmodium (Hatano and Oosawa 1971, Achenbach et al. 1979, Kukulies et al. 1983, Kukulies and Stockem 1985). On the other hand, the caffeine does not inhibit the fibrillogenesis and does not suppress the contraction activity in the droplets. The drug influences only the functional interactions between the plasma membrane and the contraction system beneath it (Achenbach et al. 1979), which is of greatest interest to the aspects of membrane-cytoskeleton dynamics approached in this study.

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Materials and Methods

Plasmodia of *Physarum polycephalum* were cultivated according to the method of Camp (1936). The veins of plasmodia migrating on the substratum were punctured with a steel needle. The protoplasm flowed out through the puncture and formed a clot on the veins surface. The clots were left on the plasmodial strands for 10 min and then immersed in the 5 mM caffeine solution in distilled water. After 10–15 min incubation at room temperature numerous spherical droplets of protoplasm were formed. Their behaviour (the internal motility) was observed in the caffeine solution either alone or with 5–10 mM $CaCl_2$.

In order to promote a more intense separation of the submembraneous contractile layers, the caffeine droplets were incubated in some organic solvents known as local anaesthetics: 3% ethanol, 5 mM benzamide and 100 mM dimethyl sulphoxide (DMSO).

Pinocytotic activity was induced by application of 50-100 mM NaCl, or 20-40 mM KCl in distilled water and the anaesthetics mentioned above. The inducers were added to the caffeine solution containing the plasmodial droplets.

The Biolar microscope with the differential interference contrast was used for observations, taking pictures and cinematography. The 16 mm films were recorded at the speed of 4 frames per second with a combined Bolex and Robot equipment.

Results

The protoplasmic droplets freshly generated from plasmodial veins by the caffeine treatment were rounded forms, 50-300 μM in diameter. Their motile activity was reduced, under the influence of the caffeine, to the internal movements (without locomotion). Morphologically they were much alike the spherical cells of *Amoeba proteus* preincubated in high temperature and called the hyalospheres by Grębecki and Kwiatkowska (1988). Like Nachmias and Meyers (1980), we observed the cytoplasmic caffeine droplets in three states: (1) relaxed, (2) active and (3) contracted ones (Pl. I 1). In the relaxed forms the granular cytoplasm uniformly filled the entire droplet (Pl. I 1a). The active state of spheres was characterized by spontaneous and regular motion of the central granular cytoplasm. The granules situated laterally on the granuloplasm periphery were flowing forth into the surrounding hyaloplasm, while the others formed an axial backward stream returning to the central mass (Pl. I 1 b). In the contracted droplets the cytoplasm was also separated into the granuloplasmic core and surrounding clear hyaloplasm. The granulo-hyaloplasm border in them was distinct and rather stable, because there was no internal circulation inside the central mass in that case (Pl. I 1 c).

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The state of droplets was not immutable, but it might change with time of incubation in the caffeine solution. The relaxed droplets had the ability to transform into the active or contracted ones. We did not observe, however, any inverse transformation leading from the contracted drops back to the active or relaxed forms.

The contracted droplets with distinctly marked stratification into the granuloplasm and hyaloplasm were used for experiments. A spontaneous detaching of an optically more dense peripheral layer from the plasma membrane and its centripetal shifting took place in such spheres. The separation of submembraneous hyaloplasmic sheets could occur along the major part of the droplet's periphery, when the granuloplasm was situated in the centre of the sphere (Pl. I 2 a). In other cases, when the granuloplasmic core was asymmetrically located, approaching by one side the drops perimeter, the detachment of cortical hyaloplasm occurred at much smaller area at the opposite pole (Pl. I 2 b, c). The separated peripheral layers of the hyaloplasm moved toward the border of granuloplasm, where they disappeared. Sometimes, however, they were not dissociated, but accumulated one on the other in the form of a pile, near the granuloplasm perimeter (Pl. I 3). Occasionally, the successively detached cortical sheets pushed toward the granuloplasm some inclusions situated inside the hyaloplasmic ring just below the plasma membrane, for example the vacuoles (Pl. II 4).

The separation of the peripheral hyaloplasm layer from the plasma membrane was a serial phenomenon. Several, sometimes above ten successive sheets were formed beneath the plasmalemma in the same region of the droplet. Their formation was regularly repeated. The time needed by each successive sheet to be detached and transported from the plasmalemma down to the granuloplasm border was nearly constant and characteristic for each droplet (with differences not exceeding 1-2 s). It ranged within 6-15 s, depending on the individual variation among the observed droplets.

The separation of the peripheral hyaloplasm layers from the plasmalemma was distinctly promoted by some anaesthetics and organic solvents, which are known to affect the membrane properties. We tested the effects of three such agents for their ability to promote the detaching and shifting the hyaloplasmic sheets from the plasma membrane. The best results were obtained after a brief (5-10 min) incubation of caffeine droplets in 3% ethanol. It resulted in doubling the number of droplets manifesting this type of cortical activity (from about 10% to 24% in the population of 104 droplets). The benzamide and DMSO had a weaker, but still positive effect of promoting the detaching of hyaloplasm. On the other hand, the frequency of the formation of the succe-

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ssive sheets separated from the plasmalemma was the same, both in the unstimulated caffeine droplets and in the droplets incubated in the anaesthetics solutions.

In the plasmodial caffeine droplets incubated in the solutions promoting the separation of the hyaloplasm (ethanol, benzamide and DMSO) sometimes we noticed the formation of pinocytotic channels or other forms of pinocytosis. The attempts to provoke pinocytosis by using its standard inducers, as KCl and NaCl, were also successfull. The administration of 20-40 mM KCl or 50-100 mM NaCl to the caffeine droplets induced the pinocytosis within a few minutes. The pinocytosis observed in the caffeine-produced droplets from *Physarum polycephalum* was comparable with this phenomenon described in the hyalospheres of *Amoeba proteus* induced by heat (Grębecki and Kwiatkowska 1988). Pinocytosis occurred only in the so-called contracted droplets, in which the protoplasmic movement was stopped. We have never noticed this phenomenon in the relaxed or active caffeine droplets. Several forms of pinocytosis were observed:

(1) Short funnel-like channels with very wide orifice, conically narrowing inside (Pl. II 5 a-c). In many cases we observed regular pulsation of the channel's conical opening (Pl. II 5 a). Presumably it reflected the rhythmic immersion and intake of the deeper segments of the channel (which were not always visible). Such pinocytosis suggested a rhythmic inflow of the liquid into the droplet. The inflow cycles were repeated many times by the same spot on the droplet's surface. It was the form of pinocytosis most often observed in the plasmodial droplets produced by the caffeine treatment.

(2) Long and narrow pinocytotic channels with weakly marked orifice (Pl. II 5 d, e). There were sometimes a few long channels present at the same point of the droplet's periphery. Several endosomes could be formation was not repeated in the same area of the droplet.

(3) Pinocytosis without channels. The spherical or other endosomes were produced just beneath the plasmalemma and were displaced across the hyaloplasmic ring toward the central granuloplasm. The formation of those endosomes was most often cyclically repeated, several times at the same point of the droplet's periphery. Several endosomes could be simultaneously seen moving one after another toward the granuloplasm (Pl. II 5 f). This form of pinocytosis was not connected with the formation of a pinocytotic channel.

We could not confirm the dependence of the form of pinocytosis on the kind of the inducer applied, as described in other cells. In the caffeine-induced plasmodial drops all forms of pinocytosis occurred in NaCl as well as in KCl solutions.

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It seems, that the localization of an area predisposed to pinocytosis was dependent on the position of the granuloplasmic core, respective to the hyaloplasmic ring. When the granuloplasm was concentrated near one pole of the droplet, the pinocytosis more often occurred on the same side, where the granuloplasm was close to plasmalemma (Pl. II 5 a, b, d). In other cases, when the granuloplasm occupied a central position, there was no favoured area of pinocytosis and it could occur everywhere along the hyaloplasmic periphery of the droplet.

Discussion

The subplasmalemmal microfilament system which is responsible for various manifestations of the tisue cell motility plays also a dominant role in the amoebae and slime moulds. It is involved in the maintenance and changes of their cell shape (Klein and Stockem 1979, Kukulies and Stockem 1985), the protoplasmic streaming (Rhea 1966, Usui 1971, Kessler 1972, Kukulies and Stockem 1985, Ishigami 1986) and endocytotic food ingestion (Stockem 1977, Klein and Stockem 1979, Taylor et al. 1980 a, b). Some of these phenomena are difficult to control in a so large system as *Physarum polycephalum* plasmodium. Therefore many experiments are performed by using as models the small plasmodial fragments: caffeine or adenine droplets (Matthews 1977, Nachmias and Meyers 1980, Kukulies et al. 1984, Kukulies and Stockem 1985), endoplasmic drops (Götz von Olenhusen and Wohlfarth-Bottermann 1979, Baranowski 1980, Kukulies et al. 1983, Kukulies et al. 1984) or thin-spread macroplasmodia (Kukulies et al. 1984, Ishigami 1986, Ishigami et al. 1981). The investigation of the spatial organization and dynamics of the cortical contractile system and its involvement in various morphogenetic and motion activities is easier in that simplified fragments of mature plasmodium.

The caffeine droplets used as a model in our experiments were obtained from the endoplasmic drops. Such drops were produced by puncturing plasmodial veins and left on the veins surface for 10-15 min. According to Baranowski (1980), in this time the endoplasmic drops organize *de novo* an extensive peripheral fibrillar system. The rebuilt actomyosin structures display a contraction behaviour characteristic for intact plasmodium (Wohlfarth-Bottermann and Stockem 1970, Isenberg and Wohlfarth-Bottermann 1976, Götz von Olenhusen and Wohlfarth-Bottermann 1979, Baranowski 1980).

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The spontaneous or stimulated separation of the peripheral hyaloplasm from the plasma membrane which has been observed by us in the caffeine droplets of *Physarum polycephalum* plasmodium demonstrates the high dynamics of the association of the filamentous layer to the droplets membrane. The actin nature of these ectoplasmic sheets detached from the inner side of the droplets membrane appears evident in light of the earlier results of Kukulies et al. (1983, 1984, 1985) who demonstrated that in the caffeine droplets the microfilamentous actin, identifiable by electron microscopy and immunofluorescence, is accumulated on the hyalo-granuloplasm border, while new actin layers are formed on the cytoplasmic side of the membrane. So, the centripetal transport of successive contractile sheets from the droplets periphery across the hyaloplasmic ring, as described *in vivo* in the present study, completes the picture.

Kukulies et al. (1984) also described the detaching of a fluorochromed actin layer from the plasma membrane and its movement inward, which resulted in filtration of peripheral hyaloplasm and its separation from central granuloplasm, in caffeine droplets and endoplasmic drops of *Physarum*. However, in this instance, the phenomenon had to be stimulated by an increase of the external Ca^{++} concentration, and just one contraction of the whole droplet could be produced in each case. In contrast to these earlier results, we were able to demonstrate that: (1) The detachment of cortical filamentous layer from the membrane may be spontaneous, (2) it can be serially repeated during long periods of time with a frequency characteristic for the droplet, (3) this type of cortex dynamics is promoted in the presence of some local anaesthetics which affect the stability of membrane-cytoskeleton linkage.

Analogous phenomenon of rhythimc detaching and shifting an optically more dense layer from the plasmalemma toward the granuloplasmic core was described by Grębecki and Kwiatkowska (1988) in the heat-shocked amoebae. Examination of this layer by electron and fluorescent microscopy (Kłopocka et al. in press) showed, that the layer is made up of the network of actin filaments and its rhythmically repeated movements probably depend on their contractile activity. Also Heath and Dunn (1978) and Heath (1983) noticed the birefringent arcs, formed near the leading edge of the lamella of migrating fibroblasts. These crescents moved back toward the nucleus, where disappeared. Similar structures have been described also in spreading epithelial cells (Masayoshi and Sato 1978) and spreading fibroblasts (Sorrano and Bell 1982). The last authors interpreted these moving arcs as "condensations of a microfilament network that move toward the nucleus as compressional waves". All mentioned observations

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showed that repeated detaching of the actin layer from plasmalemma and its shifting toward the cell centre is a phenomenon which can be observed in various types of cells.

The pinocytosis has been observed in the plasmodial caffeine droplets for the first time. It seems also to depend on the high dynamics of the filamentous layer associated with the cell membrane of droplets. It is well known, that microfilaments are involved in endocytosis as well in the tissue cells as in amoebae (see Allison 1973, Stockem 1977). The pinocytosis described here resembled this phenomenon in heat-shocked amoebae (Grębecki and Kwiatkowska 1988) and in untreated *Amoeba proteus*. During the induced pinocytosis amoeba changes its shape and becomes spherical. This general process, as well as the invagination of each channel, seem to be correlated with the contractions of the cortical layer, shifting toward the cell centre (Klein and Stockem 1979, Stockem 1979). The formation of the pinocytotic channel can be repeated many times in the same area of the droplet. This fact is also in agreement with the serial formation of channels and cortex retraction in the hyalospheres of *Amoeba proteus*. It seems that the further examination of pinocytosis in the caffeine-derived droplets of slime mould protoplasm should give a valuable contribution to the biology of *Physarum*, because as yet it could never been successfully observed *in vivo* in a normal large plasmodium.

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EXPLANATION OF PLATES I–II

1: Three forms of plasmodial protoplasm droplets obtained by incubation of large endoplasmic drops in 5 mM caffeine solution: a — relaxed state without internal stratification, b — state with active granuloplasm (arrows), c — presumably contracted state with full and stable separation of the granuloplasm (G) and hyaloplasm (H)

2: Detaching of the optically dense layers of peripheral hyaloplasm from the plasmalemma and their retraction toward the granuloplasm (arrows), induced by 3% ethanol

3: Successive layers of the peripheral hyaloplasm retracted to the hyalo-granuloplasm border (double arrows in a), and accumulated in the form of a pile (bundle of arrows in b)

4: A vacuole pushed by the successive contractile layers of hyaloplasm (arrows in a,c,f) from the plasmalemma toward the droplet centre

5: Pinocytosis induced in the caffeine-derived plasmodial droplets by 20–40 mM KCl (a,e,f) or 50–100 mM Na Cl (b,c,d): a — rhythmically pulsating orifice of a channel (arrow-head), b, c — large conical channels with funnel-like openings (arrow-heads), d, e — long and thin type of channels (arrows), f — serial intake of endosomes (arrows) without developing a continuous channel. Calibration bar 100 μm in all pictures

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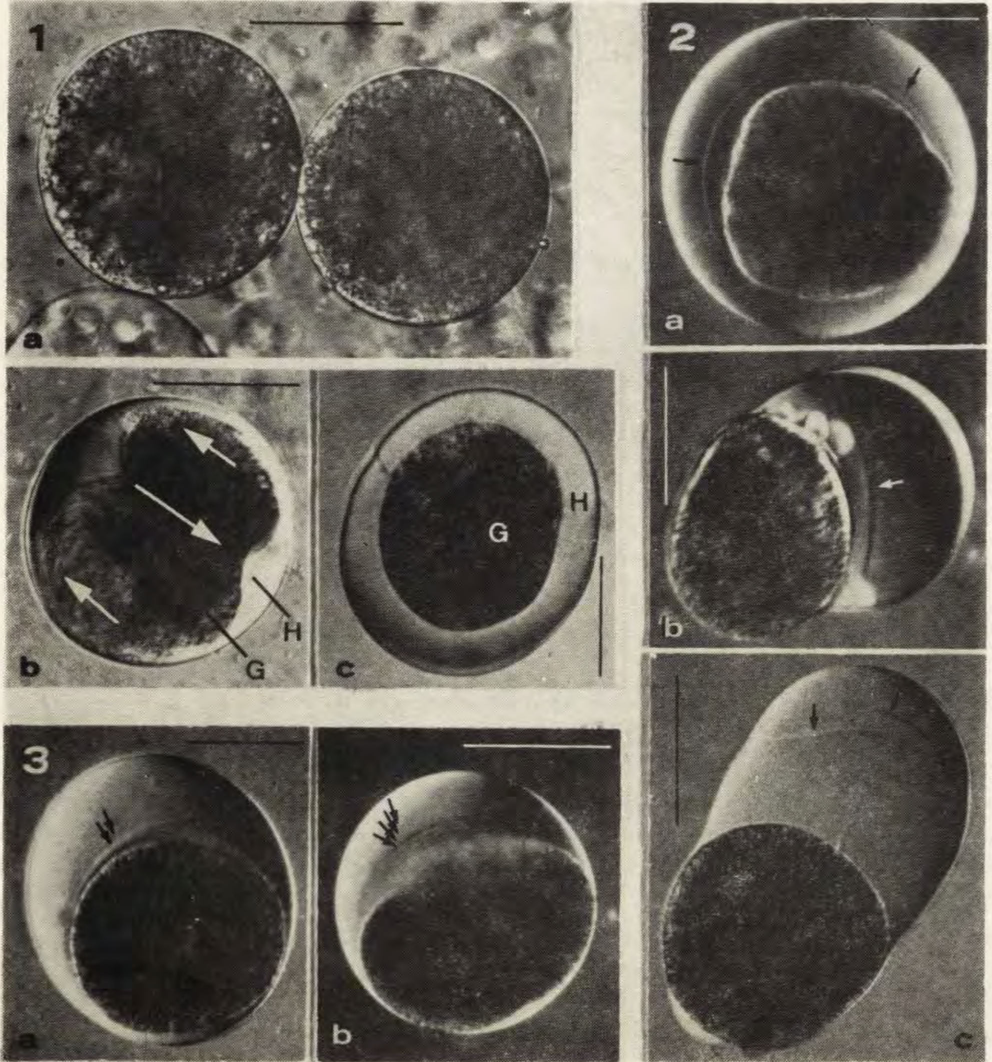

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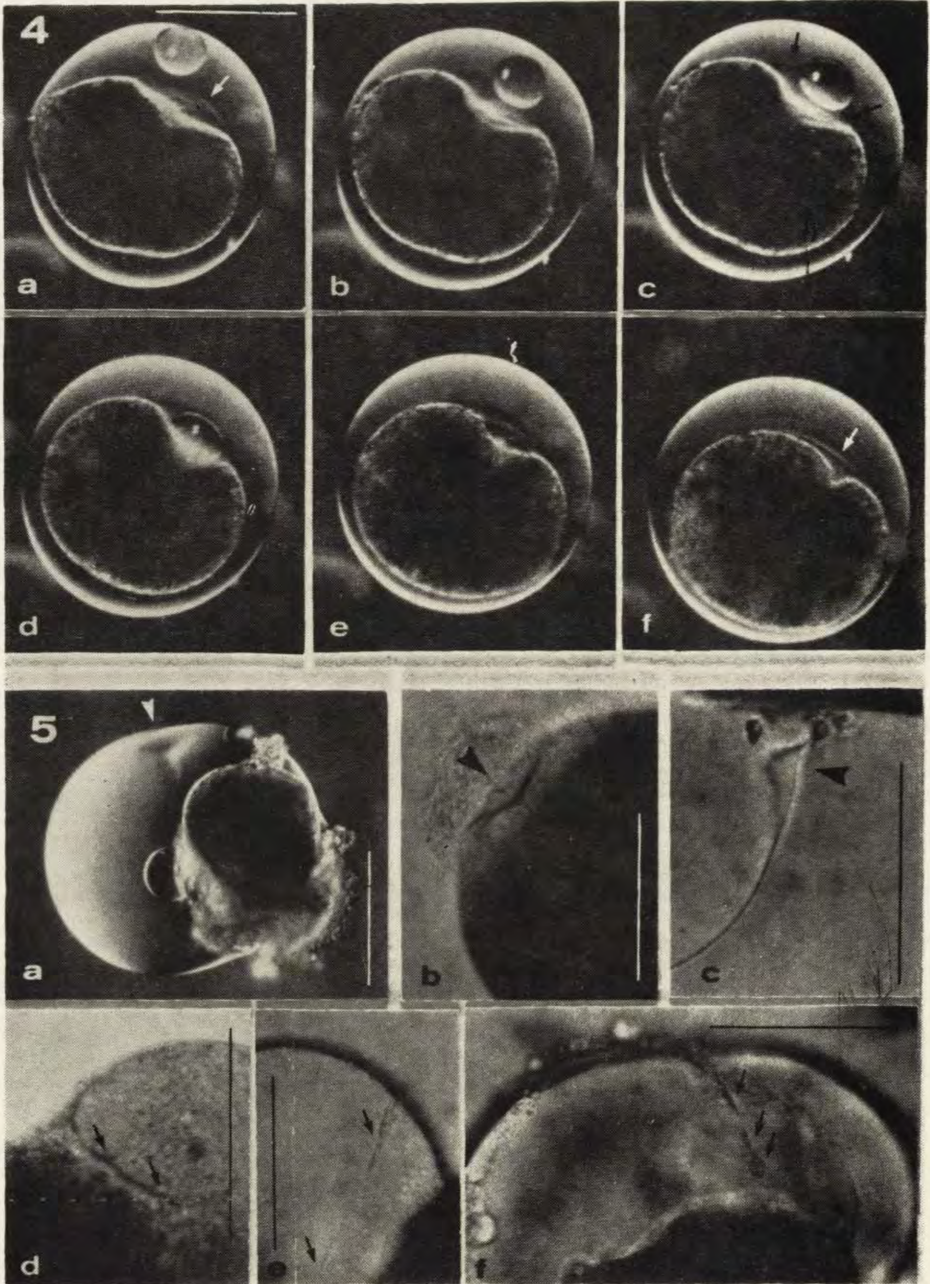

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Characterization and Partial Purification of Mating Pheromone Excreted by Mating Type II Cells of the Ciliate *Dileptus anser*

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Synopsis. Mating pheromone (MP) excreted by mating type II (MT II) cells of the ciliate *Dileptus anser* was characterized and partially purified. At a concentration lesser than 10^{-9} gramm per milliliter partially purified MP II specifically induces the sexual behaviour of complementary mating type cells (MT I or III) and their conjugation with MT II cells. MP II shows thermostability (100°C — 30 min) in a acidic (2–4) and neutral pH of the medium. It was identified as a polypeptide (its activity was fully destroyed by some proteases whereas RNAse and DNAse and micrococcal nuclease did not affect it). Thin layer chromatography of cell free fluid (CFF) from MT II cells on Silufol plates (piridine — acetic acid — n-butanol — water 40:14:68:25) revealed the presence of 5 spots stained with ninhydrin. The only component possessing MP II activity had low mobility (R_f = 0.08) in this system. Rather an efficient method for concentrating and desalting MP II was a pressure ultrafiltration of CFF (MP II fails to pass through the Amicon UM 05 membrane). By gel filtration of the CFF concentrate on a Bio-Gel P-6 column MP II was 100-fold purified. Its molecular weight was defined by the gel filtration method to be ca. 3000 Da.

Ciliates are among most promising objects for studying the evolution of intercellular communication and recognition mechanisms (Luporini and Miceli 1986 a). In these unicellular animals the sexual process occurs during cell conjugation. In a great number of species some stages of interaction between individuals leading to the formation of pairs are highly specific, in a sense they are realized only between

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ciliates that belong to different (complementary) mating types (Grell 1973). In some of the species studied preconjugational interactions are mediated by specific substances excreted to medium which are known as mating pheromones (MPs) (Luporini and Miceli 1986 b). The presence of MPs was revealed in several species of *Blepharisma (B. japonicum* — Miyake 1968, *B. americanum, B. musculus, B. stoltei, B. tropicum* — Miyake and Bleyman 1976, *Blepharisma* sp. — Ricci and Esposito 1981), in some *Euplotes* species (*E. patella* — Kimball 1942, Katashima 1959, 1961, Akada 1985, *E. eurystomus* — Katashima 1959, *E. octocarinatus* — Heckmann and Kuhlmann 1982, *E. raikovi* — Luporini et al. 1983), in *Oxytricha bifaria* (Esposito et al. 1976), in *Dileptus anser* (Tavrovskaja 1979), in *Tokophrya infusionum* and *T. lemnarum* (Sonneborn 1978).

Biochemically, MPs were first investigated in *Blepharisma japonicum*. One of two MP types detected in this ciliate is presented by a basic glycoprotein with the molecular weight of about 20 kDa (Miyake 1974 a, b, Miyake and Beyer 1974) and the second, by a low molecular substance, a derivative of tryptophane — Ca-3-(2′-formyl amino-5′-hydroxybenzoil)-lactate (Braun and Miyake 1975, Entzeroth and Jaenicke 1981). The latter MP affects other *Blepharisma* species too while the high-molecular MP is species specific (Miyake and Bleyman 1976). In the last few years a study has been made of MPs in other ciliates having multiple mating types. Thus it has been shown that two from four different MPs in *Euplotes patella* of syngen 2 are low-molecular proteins that behave similarly during electrophoresis in gels (Akada 1986). In *E. octocarinatus* all four MPs are proteins (glycoproteins?) with the molecular weight of ca. 20 kDa (Miyake and Nobili 1984, Weischer et al. 1985, Schulze Dieckhoff et al. 1987). In *E. raikovi* MPs are presented by a family of homologous proteins (M.w. 10-12 kDa) (Luporini et al. 1986, Luporini and Miceli 1986 b, Raffioni et al. 1987).

Such investigations should be expanded to other ciliate species excreting MPs because we want to know how systems of mating types originated and developed in ciliates and how MPs and mechanisms of their reception evolved. A most promising new object for such studies is *Dileptus anser*, another ciliate species from a primitive group *Gymnostomatida*. This fact is of evolutionary importance because all previously reported species that excrete MPs, belong to two systematically distant and evolutionary more advanced groups *Heterotricha* and *Spirotricha*. *D. anser* has only three mating types and each of them excretes its own MP. The MP activity can be detected through the use of various biolo-

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gical reactions induced in the cells by heterologous culture media (Tavrovskaja 1974, Afon'kin and Skovorodkin 1986, Afon'kin and Yudin 1986). The present communication gives a preliminary biochemical characterization of one of the three MPs in this species.

Materials and Methods

Clones of *Dileptus anser* were cultured according to Nikolaeva (1968) at 25°C. To obtain cell-free culture fluid (CFF) a mass culture (about 150,00 cells per litre) was first grown in vessels (0.5 l). To get rid of debris and food organisms (*Tetrahymena pyriformis* GL) dileptuses were transferred carefully to a fresh culture medium (Prescott-Carrier salt solution — Prescott and Carrier 1964). For this purpose *D. anser* mass cultures were poured into narrow-necked glass flasks (1–2 l) and 1–2 h later ciliates accumulating in the upper part of the flask were sucked out using a pipette. The mechanism of this reaction of dileptuses is not known (negative geotaxis?) but it may be used for collecting or partial concentrating ciliates which do not stand centrifugation.

A greater part of the cells kept in a fresh portion of medium during 24 h without food was removed for further growth. The medium the dileptuses were taken from, with few remaining cells and excreted pheromone was filtered through cotton wool and then through cellulose filters to obtain CFF.

As a source of MP clone No. 20 (MT II) isolated from nature in 1984 was used (Yudin and Afon'kin 1987). According to the preliminary data (Tavrovskaja 1974) supported by our experiments MP of this type is a thermostable substance.

Cell-free fluid (CFF) showing MP II activity was 50-fold concentrated in a rotation evaporator under low pressure at 65°–70°C during several hours. A small precipitate was removed by centrifugation (20 min — 100,000 g). Thus cleared supernatant could be stored frozen for 1 or 2 month without essential change in MP II activity. In some of the experiments the CFF supernatant was further concentrated and desalted by the pressure ultrafiltration method to a small volume (MP II activity was retained by the Amicon UM 05 membrane).

Thin-layer chromatography of CFF concentrate desalted by ultrafiltration was carried out on Silufol plates (ČSSR) in the mixture of piridine — acetic acid — n-butanol — water (40:14:68:25). Peptide components were detected by the ninhydrin method (0.2% ninhydrin in n-butanol — acetic acid 95:5) with subsequent heating at 140°C (Dévényi and Gergely 1974). To identify MP II immediately after chromatography the Silufol plates were cut into several zones corresponding to the peptide components revealed with ninhydrin. The material was eluted by the water (1 ml) during 15 h (at 25°C) and assayed biologically for MP II activity.

The method of enzymatic destruction was used to identify the MP II nature. CFF concentrate was treated by various proteolytic enzymes in the concentration range of 0.5–2.0 mg per ml during 2 h at 37°C and pH 7.2. The raction was stopped by boiling of the medium for 10 min since the latter procedure did not destroy the

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MP II activity. Samples of the CFF concentrate treated with preliminarily thermoinactivated enzymes were used as a control.

Lyophilized CFF (2 mg in a volume of 100 μl) was subjected to SDS-polyacrylamide gel electrophoresis according to Laemmli (1970) with subsequent Coomassie Brilliant Blue staining.

The protein content was determined according to Lowry et al. (1951) or using the microbiurhet method (Dévényi and Gergely 1974).

Gel filtration of the CFF concentrate was performed in a Bio-Gel P-6 columm (14 × 830 mm). Elution medium contained 20 mM Tris HCl pH 7.2 plus 50 mM NaCl, the flow rate being 9 ml/h. The volume of fractions was 2 ml. The protein content of the fractions was determined simultaneously by several methods — by light absorption at 230 and 280 nm, according to Lowry et al. (1951) and by microbiurhet technique (for identification of peptides of various amino acid composition). Before fractions were tested for pheromone activity they were diluted preliminarly 30–100-fold since 2 mM NaCl is toxic for cells. The pheromone molecular weight was determined under the same conditions on a column calibrated with appropriate molecular weight markers.

For determining the MP activity in CFF and its fractions one of the effects produced by pheromone was utilized, namely ability to induce additional "conjugational" divisions in cells of complementary mating type (Tavrovskaja 1974). Ten cells from a tester clone (one day after feeding) in their "own" culture medium were placed into each of the 6 microplate wells (Afon'kin and Yudin 1985) thereupon fluid to be tested was added its concentration being diluted twofold. In 18–20 h the number of cells was counted in the microplate wells and thus the percent of cells that had undergone division was determined. There was a control for each experimental series (6 microplate wells each containing 10 cells in their "own" culture fluid). "Chi-square" test was applied to evaluate the significance of differences between the experiment and the control at a 5% significance level.

Results and Discussion

A 10-50-fold CFF concentrate is a viscous yellowish fluid of acidic pH (5.4-6.1). The protein content of CFF detected according to Lowry et al. (1951) is 1-1.5 μg/ml being consistent with values given in literature for other ciliate species excreting mating pheromones (Luporini et al. 1986). Biological activity of a 10-fold CFF concentrate could be demonstrated even at its 2000-fold dilution (10^{-8} g/ml protein concentration) (Fig. 1).

To test the specificity of MP II excreted by clone No. 20 we studied its biological action on various mating type cells (clones No. 11, 52, 59 — mating type I; clones No. 19, 20, 61, 73 — mating type II; clones No. 8, 28 — mating type III). Actually, the MP II action was highly specific and was displayed only with regard to complementary mating type cells (mating types I and III). Various clones of the same mating type

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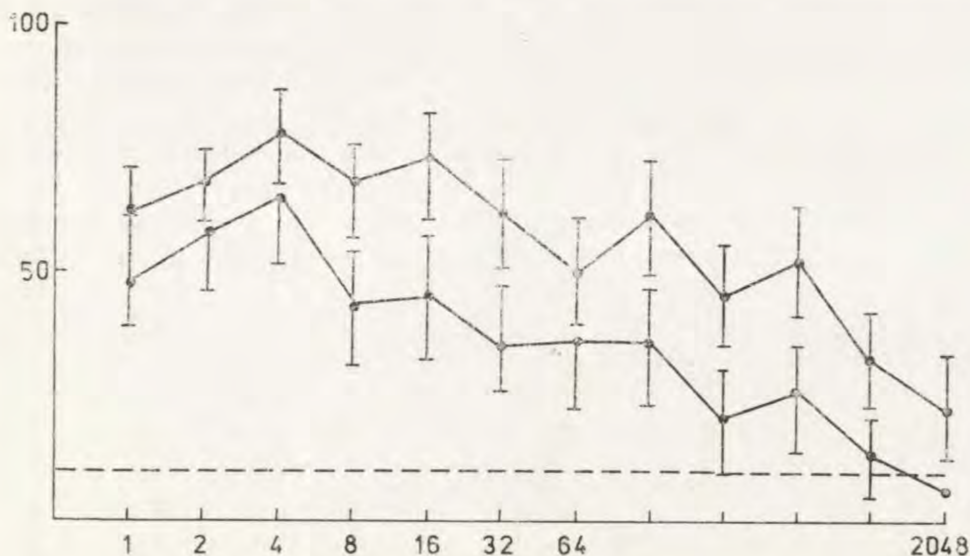

Fig. 1. Biological activity of 10-fold CFF concentrate (clone No. 20) at successive dilutions
Abscissa, CFF dilutions (0-, 2-, 4-fold, etc.); *ordinate*, percent of cells that underwent division Tester clones No. 11 (1) and 52 (2). Vertical bars, 95% confidence intervals; dashed line, statistically significant difference from the control in this experiment

differed in their sensitivity to MP II (Fig. 1), clone No. 11 being more sensitive compared to clone No. 52. The action of MP II on complementary mating type cells was dose-dependent with a pronounced saturation effect (Fig. 1). Its activity fully retained upon 500-fold dilution of CFF concentrate and lowered with further dilution. The saturation effect and high sensitivity seems to indicate the presence of a receptory stage in the action mechanism of MP II. A similar suggestion was made for MPs of other ciliate species.

MP II appeared to be a thermostable substance: it did not lose its activity after boiling (100°C — 30 min) in an acidic (2-4) and neutral pH of the medium, whereas in basic pH (8.5-10.0) its activity was fully destroyed.

Dialysis through cellophane bags as well as lyophilization of CFF resulted in significant (more than 100-fold) loss of MP II activity.

To identify the chemical nature of MP II the method of enzymatic destruction was applied. It was shown that the treatment of CFF by various proteases (trypsin, pronases P and E, proteinase K) results in a complete loss of MP II activity. At the same time the treatment with nucleases (DNAse, RNAse, micrococcal nuclease) and hyaluronidase did not change its activity. These data allow the conclusion that MP II has

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a peptide nature, its molecular weight is probably small since MP II is a dialyzable substance. The conclusion is supported by results of the CFF lyophilisate SDS-electrophoresis in 12% polyacrylamide gel with subsequent staining with Coomassie Brilliant Blue. Since no protein bands were detected with this method, it was concluded that proteins from CFF of the clone 20 had molecular weight smaller than 10 kDa.

Thin-layer chromatography of CFF concentrate enabled to demonstrate the heterogeneity of its peptidic composition (Fig. 2). By means

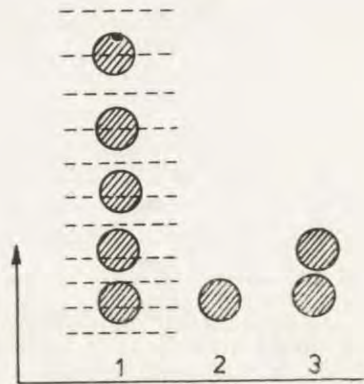

Fig. 2. Thin-layer chromatography of CFF concentrate after pressure filtration through Amicon UM 05 membrane on Silufol plates stained with ninhydrin Dashed lines designate zones in chromatogramme cut out for elution for the material and its subsequent tests for MP II activity. 1 — CFF concentrate, 2 — rechromatography of MP II active material, 3 — chromatography of partially purified MP II material

of ninhydrin staining five coloured spots ($R_f = 0.08$-0.35) were detected. After chromatography (without any staining) and evaporation of organic solvents the material contained in the detected zones was eluted with water and tested for its biological activity. The MP II activity was revealed only in a low mobility fraction ($R_f = 0.08$) stained violet with ninhydrin (Fig. 2). Hence, not all peptide components in CFF have MP activity and the problem is to purify MP from the CFF original material. It must be specially emphasized that prior to determination of biological activity we had to dilute significantly eluates from separate zones because of the toxic effect of organic solvents. This does not rule out the existence of other regions of lower MP activity not detectable by our test.

Concentration of the CFF to a small volume was the first step in purification of MP II. It presented a problem since MP II was a dialyzable protein (through cellophane bags that were at our disposal) and lost its activity during lyophilization. This inactivation was probably

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due to various salts that are contained in culture medium and, therefore, in CFF (8 μM $MgSO_4$, 30 μM $CaHPO_4$, 80 μM KCl, 90 μM $CaCl_2$, 300 μM NaCl — Prescott and Carrier 1964). Their concentration obviously increased during concentration of the CFF.

The CFF ultrafiltration through Amicon UM 05 membrane proved to be an adequate method for concentrating and subsequent desalting MP II (MP II itseelf was retained by the membrane). The procedure of MP II purification was as follows. CFF (20 l) was concentrated at reduced pressure in a rotatory evaporator to 0.5 l at 65°-75°C. Further concentration was achieved by ultrafiltration through Amicon UM 05 membrane to 10 ml at +4°C. A portion of the material obtained (2 ml) was chromatogra-

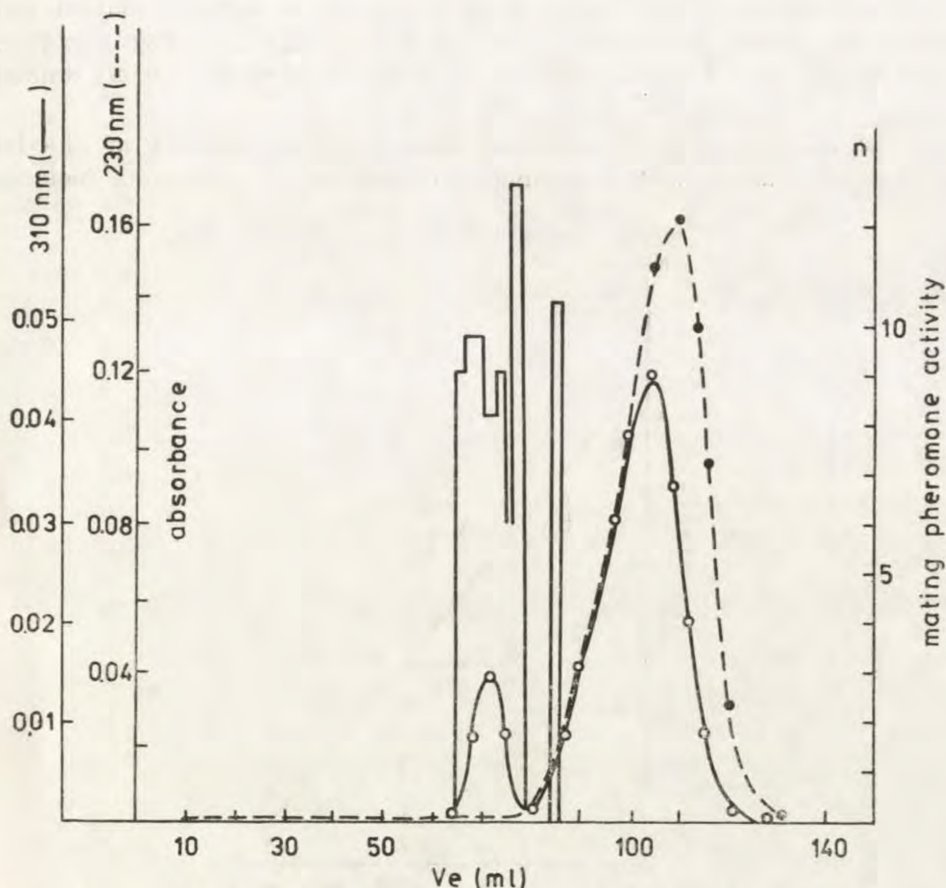

Fig. 3. Bio-Gel P-6 elution pattern of CFF concentrate obtained by ultrafiltration *Abscissa*, elution volume; *ordinate*, absorbance at 230 nm and protein content determined by the biurhet method (left), number of additional ("conjugational") divisions (right). Dashed line, elution pattern (absorbance at 230 nm); solid line, protein content; columns, MP II activity

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phed on a Bio-Gel P-6 column (separation limits 1000-6000 Da). Elution medium contained 20 mM Tris HCl pH 7.2 plus 50 mM NaCl. The chromatography made it possible to separate effectively fractions with the MP II activity from the main protein peak (Fig. 3). It should be noted that fractions in the MP II activity peak failed to stain after Lowry et al. (1951) and showed no absorbance at 280 nm. These results suggest that MP II has a low content of aromatic amino acids. All the material stained according Lowry et al. (1951) and characterized by absorbance at 280 nm displays no MP II activity. The degree of MP II purification achieved by gel filtration on Bio-Gel P-6 was more than 95% (based on protein content).

The fractions in the MP II activity peak (V_e = 65-75 ml) were pooled, concentrated on air, dialyzed 1 h against water to low salt content and chromatographed on Silufol plates as described above. Two spots — one major (R_f = 0.17) and the other suppplementary (R_f = 0.09) stained violet by ninhydrin were detected.

The MP II molecular weight estimation was carried out on Bio-Gel P-6 column which was preliminarily calibrated with adequate molecu-

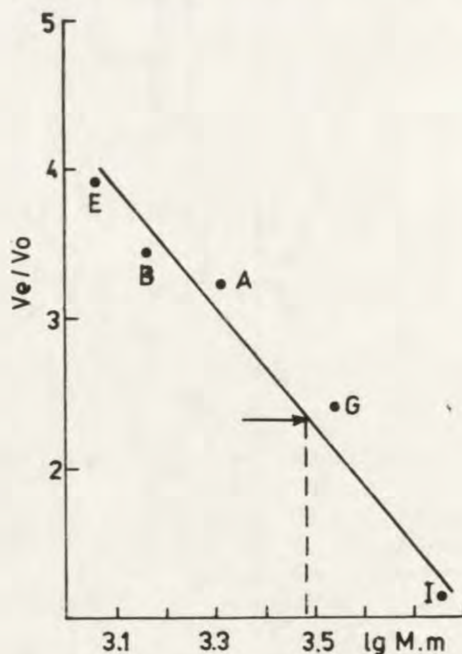

Fig. 4. MP II molecular weight determination on Bio-Gel P-6 column. Molecular weight markers used: E — eledoisine (1190 Da), B — bacitracin (1450 Da), A — apamin (2030 Da), G — glucagon (3500 Da), I — insulin (5600 Da)
Abscissa, lg M.w.; *ordinate*, V_e/V_o; V_o aws evaluated using Dextran Blue

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lar weight markers (eledoisine — 1190 Da, bacitracin — 1450 Da, apamin — 2030 Da, glucagon — 3500 Da, insulin — 5600 Da). The value obtained is ca. 3000 Da (Fig. 4). It should be noticed that with increase in the NaCl content in the elution medium to 200 mM supplementary peaks of the MP II activity with a smaller molecular weight (1450 and lesser) appeared. It may therefore be assumed that MP II is represented by some peptide monomeres capable of associating and dissociating under different ionic conditions without a significant loss of its biological activity.

In some experiments when testing fractions most active in respect of inducing cell divisions, selfing pair formation was observed. Such pairs were never noted with fractions devoid of division-inducing activity. This observation suggests that factors inducing selfing and conjugational divisions are the same thing. At the same time, we failed to observe pair formation when diluting the fractions proved to be active for selfing, though cell divisions were still induced in several successive dilutions. It suggests that higher concentrations of the pheromone are required for selfing than for cojugational cell divisions. Similar observations were made with other two mating pheromones of *D. anser*; at present their isolation and partial purification are nearing completion.

Conjugational divisions are likely to be necessary to synchronize cells that come into sexual interaction. However, the induction of such cell divisions with mating pheromones seems to be unique for *D. anser* and was never reported for other pheromone-excreting ciliates. It favours the hypothesis that mating pheromones might originate from autocrinous cell regulators (Luporini and Miceli 1986 b).

To summarize, the mating pheromone of type II (MP II) excreted by the ciliate *Dileptus anser* (clone No. 20 belonging to mating type II) and affecting specifically the complementary mating type cells (I and III) is a thermostable in acidic and neutral pH polypeptide with the molecular weight ca. 3000 Da. The latter value is significantly lower than the molecular weights of other known proteinaceous mating pheromones excreted by other ciliate species. Biochemical characterization of two other mating pheromones of *D. anser* (MP I and III) will enable us to compare the system of intercellular communication in this species with that of other ciliates using mating pheromones.

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Cytochemical Study of Dehydrogenase Activity in Two Euglenid Species of the Genus *Parastasia* Michajłow, 1966

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Synopsis. The presence of succinate dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, lactate dehydrogenase, α-glycerophosphate dehydrogenase, β-hydroxybutyrate dehydrogenase and glutamate dehydrogenase in *Parastasia fennica* and *P. macrogranulata*, the *Euglenida* infesting the alimentary canal of copepods, was investigated using cytochemical methods. Differences in the activity of enzymes of the citric acid cycle and of the glycolytic pathway discovered in the endogenous and exogenous phases of the parasites' life-cycles suggest a flexibility of cell oxidative metabolism in both *Parastasia* species, which may be either aerobic or anaerobic depending on the life-cycle phase and the environmental oxygen content.

Knowledge of metabolism and its regulation in the cells of endoparasitic *Euglenida* which infest the copepod alimentary canal is lacking. Studies on the morphology and life-cycles of *Parastasia fennica* (Michajłow 1966) and *P. macrogranulata* Wita, 1985 have demonstrated that large amounts of paramylon collect in the cytoplasm of the cells of the two species. This polysaccharide, which is specific for *Euglenida*, constitutes the essential reserve substance, being the main energy source for both species. The paramylon stored in the course of these trophozoites' endoparasitic life is intensively utilized at the time of palintomic division and the emergence of flagellate forms in the external environment. The trophic (endogenous) and reproductive (exogenous) phases of the life-cycles of *P. fennica* and *P. macrogranulata* occur in environments differing in oxygen content. The aim of the present study was to identify the distribution and cytochemical activity of certain enzymes of the oxidoreductase class in the cells of both *Parastasia* species.

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Material and Methods

The study included two species of the genus *Parastasia* Michajłow, 1966, namely *Parastasia fennica* and *Parastasia macrogranulata*. They are endoparasites of the copepod alimentary canal and their reproductive phase occurs outside the host (Michajłow 1966, 1978, Wita 1984). The copepods were collected from small reservoirs of still water in the area of Leningrad in the summer of 1984.

Using the commonly employed methods of cytochemical dehydrogenase detection described by Barka and Anderson (1963), and Pearse (1972) the presence of the following coenzyme — linked dehydrogenases was demonstrated: malate dehydrogenase (NAD), isocitrate dehydrogenase (NAD, NADP), β-hydroxybutyrate dehydrogenase (NAD), α-glycerophosphate dehydrogenase (NAD, NADP).

Succinate dehydrogenase was detected according to the method of Nachlas et al. (Nachlas et al. 1957) as modified by Barka and Anderson (Barka and Anderson 1963). Lactate dehydrogenase (NAD) was revealed by means of a method originally proposed by Jacobsen (Jacobsen 1969, Beyer et al. 1977 b, c, 1978 a, b), The basic incubation solution for all enzymatic reactions contained nitro-blue tetrazolium.

The enzymes were detected in intact trophozoites immediately after their removal from the host gut well as in consecutive stages of palintomic division and in flagellate forms resulting from palintomy. The organisms were placed on microscopic slides, dried at room temperature and then incubated in acetone at 0°C and in chloroform at −15°C to remove lipids from the cells, which distort the normal picture of the histochemical reaction (Jacobsen 1969, Beyer et al. 1977 a–c, 1978 a, b).

The dehydrogenases under study were investigated simultaneously in all developmental stages of each species in order to obtain comparable results. Incubation was carried out at room temperature (for all enzymes) for 30 min, which was established after a series of trials as the optimum period for the studies in question. A longer incubation was found to result in a too intense background staining. The incubation solution was used with or without phenazine methosulphate (PMS). After incubation, the cells were fixed in a 15% formalin solution, rinsed in water and stained, if necessary, with neutral red or methyl green. Subsequently the specimens were embedded in glycerol — gelatine or, after dehydration, in Canada balsam. Control specimens were incubated in solutions free of the substrates. The incubation solution was checked on mouse liver cells placed at the other end of the microscopic slide.

The cytoenzymatic results were interpreted by comparing the intensity of formazane precipitation, an indicator of enzyme activity, in the experimental cells. Drawings were made using a camera lucida with a 15× eyepiece and 40× objective. Photographs were taken with the aid of a Zetopan light microscope under normal lighting conditions. A detailed study of dehydrogenase localization was carried out under a microscope with a 20× eyepiece and 90× and 100× objectives.

Results

Parastasia fennica. The following enzymes of the citric acid cycle were found to be active in this parasite species: succinate, malate and isocitrate dehydrogenases.

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Trophozoites studied immediately after removal from the host gut strongly demonstrated succinate dehydrogenase activity (Pl. I 1 a). A particularly strong reaction was observed in the cells undergoing division (Pl. I 1 b), in the cytoplasm of which numerous formazane granules could be seen filling the entire cell except the nucleus. The flagellate forms, soon after their emergence, displayed an equally strong reaction to succinate dehydrogenase (Pl. I 1 c), although the reaction gradually became weaker as the organisms stayed in water and used up paramylon and other reserve substances, which enable them to live in the external environment. The flagellate forms live in water for a few days and then perish, unless they have been ingested by the host in the meantime. Dying organisms displayed an almost total absence of succinate dehydrogenase activity.

The activity of malate dehydrogenase in trophozoites removed from the host gut was also pronounced (Pl. I 2 a). The higher enzyme activity was displayed by the organisms undergoing palintomic division (Pl. I 2 b) as well as by the descendant flagellate forms (Pl. I 2 c). The malate dehydrogenase activity increased in the course of *P. fennica* development in the outer (aerobic) environment and subsequently diminished in dying flagellate forms not swallowed by their host.

In trophozoites (Pl. I 3 a) studied immediately after their removal from the host gut, isocitrate dehydrogenase activity was less pronounced than in cells undergoing division by palintomy (Pl. I 3 b). A similarly intense reaction was observed in the invasive flagellate forms in the initial period following their emergence. With time, as they inhabited water, the enzyme activity gradually weakened and in dying organisms, which had not been swallowed by the host, isocitrate dehydrogenase disappeared.

The activity of lactate dehydrogenase was established in all stages of the *P. fennica* life-cycle, with the exception of the flagellate forms. The most pronounced reaction was elicited in trophozoites, both young and mature, soon after their removal from the host gut (Fig. 1 a, Pl. II 4 a). In trophozoites remaining in water for about 24 h prior to palintomic division, lactate dehydrogenase activity was lower (Fig. 1 b). Only a trace activity of the enzyme was found in the invasive flagellate cells during the entire period of their life in water (Fig. 1 c).

The activity of α-glycerophosphate dehydrogenase was investigated in trophozoites of various sizes immediately after their removal from the host gut as well as in the cells in the course of their palintomic division and the descendant flagellate forms. The α-glycerophosphate dehydrogenase activity in trophozoite cells was comparatively high and expressed by a considerable number of formazane granules filling the

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entire cell (Pl. II 5 a). In the course of their stay in the outer environment the enzyme activity decreased from moderate, in the division stages and the flagellate forms soon after their emergence (Pl. II 5 b, c), to

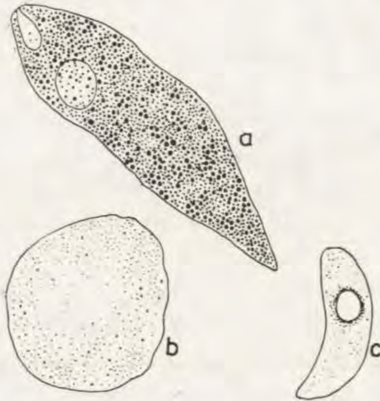

Fig. 1. Distribution of lactate dehydrogenase in *P. fennica*. a — a trophozoite immediately after its removal from the host gut, b — a trophozoite which has stayed in water for ca. 24 h, c — a flagellate form (after the last division)

Fig. 2. Distribution of β-hydroxybutyrate dehydrogenase in a trophozoite of *P. fennica* immediately after its removal from the host gut

weak in the flagellate forms which had lived in water for several days.

The activity of glutamate dehydrogenase was detected in all stages of the *P. fennica* life-cycle (Pl. II 6).

The activity of β-hydroxybutyrate dehydrogenase was high in all stages of the life cycle in *P. fennica* (Fig. 2, Pl. II 7 a-c).

Parastasia macrogranulata. The activity of enzymes of the citric acid cycle and of the glycolytic pathway was investigated mainly in trophozoites of different sizes and thus of different ages.

As in *P. fennica*, the enzymes of the citric acid cycle were studied in this species as follows: succinate dehydrogenase, malate dehydrogenase and isocitrate dehydrogenase. *Parastasia macrogranulata* cells investigated immediately after their removal from the host gut demonstrated a moderate activity of succinate dehydrogenase (Pl. III 8 a) with a subsequent marked increase of activity in the course of their stay in water. The activity became high in the pre-division forms (Pl. III 8 b) and post-division forms soon after division (Pl. III 8 c). The latter live in water for a period of time but their enzymatic activity gradually decreases as they stay longer in the outer environment and they perish after their energy reserve has been exhausted unless they have been ingested by the host (Pl. III 8 d).

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Malate dehydrogenase also demonstrated high activity. No significant difference in the dehydrogenase activity was established between the trophozoites studied immediately after their removal from the host gut and those which were investigated following a stay in water of varying duration (Pl. III 9 a-c).

The study of isocitrate dehydrogenase, the activity of which was very pronounced, yielded similar results. The entire trophozoite cytoplasm was filled with formazane granules (Pl. III 10 a-c).

Fig. 3. Presence of lectate dehydrogenase in a trophozoite of *P. macrogranulata*

Fig. 4. Distribution of β-hydroxybutyrate dehydrogenase in a trophozoite of *P. macrogranulata*

Enzymes of the glycolytic pathway in *P. macrogranulata* demonstrated a fairly low activity. Lactate dehydrogenase activity seen in the trophozoite cytoplasm was small and only a few formazane granules were seen near large paramylon grains (Fig. 3, Pl. IV 11 a-c).

Low activity is also characteristic of another enzyme of the glycolytic pathway, i.e., α-glycerophosphate dehydrogenase. Small formazane granules deposited in the trophozoite cytoplasm were scarce (Pl. IV 12 a-c). β-hydroxybutyrate dehydrogenase demonstrated a fairly high activity in *P. macrogranulata*. The position of mitochondria with clear reactions of the enzyme close to the paramylon grains is noteworthy (Fig. 4, Pl. IV 13).

Glutamate dehydrogenase in the *Parastasia macrogranulata* cell is evident, which confirms its fairly high activity in all trophozoites investigated. However, there are only a few formazane grains in the cytoplasm (Pl. IV 14).

Discussion

The present study was the first investigation ever of dehydrogenases in the cells of *P. fennica* and *P. macrogranulata*. The two *Parastasia* species selected for the study differ in a number of morphological features and the life-cycle (Michajłow 1966, Wita 1984). All the dehydro-

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genases investigated are known to constitute an important link in the reactions of biological oxidation and are the typical eukaryotic cells. In *Euglenida* they have been most thoroughly studied in *E. gracilis* (Buetow 1968, 1982, Lloyd and Cantor 1979). The present studies have revealed that the investigated enzymes of the citric acid cycle are characteristic of both the endogenous and exogenous phases of the life-cycle in the two *Parastasia* species, although particular stages within each species, and between both species differ in their enzyme activity. The enzymes are characteristically more active in the exogenous phase with all stages in an environment rich in oxygen.

Also enzymes of the glycolytic pathway, i.e., lactate dehydrogenase and α-glycerophosphate dehydrogenase, are present in *P. fennica* and *P. macrogranulata* cells throughout all stages. Differences in enzyme activity between particular developmental stages of both species are more pronounced in enzymes of the glycolytic pathway than in enzymes of the citric acid cycle. The high activity of lactate dehydrogenase and α-glycerophosphate dehydrogenase discovered in the *P. fennica* trophozoites studied immediately after their removal from the host gut shows that the glycolytic processes are very intensive. A decreased activity of the enzymes after the trophozoites have entered water is associated with an increased activity of the citric acid cycle and suggests the predominance of aerobic processes in cell metabolism.

P. *macrogranulata* cells demonstrate a lower activity of lactate dehydrogenase and α-glycerophosphate dehydrogenase than cells of *P. fennica*. A comparatively weak cytochemical reaction for lactate dehydrogenase activity was observed in the cytoplasm of trophozoites immediately after their removal from the host gut. This might suggest that, in contrast to *P. fennica,* the aerobic type of respiration predominates in *P. macrogranulata* cell metabolism. β-hydroxybutyrate dehydrogenase has been found to be highly active in the two *Parastasia* species and this tends to confirm an intensive oxidation of fatty acids.

Glutamate dehydrogenase, the more important enzyme of cell metabolism in *P. fennica* and *P. mecrogranulata,* is responsible for amino acid synthesis from keto acids and ammonia. It demonstrates high activity in trophozoites from the host gut as well as in the stages occurring in the outer environment, an increase in the activity being observed outside the host.

The findings seem to indicate that energy metabolism in the cells of *P. fennica* and *P. macrogranulata* is of a mixed nature, i.e., it involves both anaerobic and aerobic reaction sequences showing a changeable intensity rate. Such a kind of cell oxidative metabolism has been seen also in other endoparasitic protozoa that live in environments

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with a differing oxygen content (Montalvo et al. 1971, Beyer et al. 1977 a–c, 1978 a, b, Gutteridge and Coombs 1977, Marr 1980, Beyer and Sidorenko 1983, Uspienskaya 1984, McLaughlin and Aley 1985). It appears that in *P. fennica*, in the endogenous phase of its life-cycle, anaerobic respiration predominates while in *P. macrogranulata* a weak aerobic respiration is observable in the same phase. Entering water leads to the intensification of oxidative processes in trophozoites and, in this exogenous phase, in both species, aerobic respiration predominates, being more effective in supplying energy necessary for movement and proliferation.

The present findings confirm that metabolism in the *Parastasia* species under study is flexible and is adjusted to the conditions in which it occurs and to the particular phases of the life-cycles occurring in environments with a differing oxygen content.

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EXPLANATION OF PLATES I–IV

1: Succinate dehydrogenasce in *P. fennica*, *a* — a trophozoite, *b* — palintomic division, *c* — cells after the last palintomic division prior to flagellum formation.
2: Malate dehydrogenase in *P. fennica*, *a* — a trophozoite, *b* — a cell undergoing palintomic division, *c* — flagellate forms
3: Isocitrate dehydrogenase in *P. fennica*, *a* — a trophozoite, *b* — a cell undergoing division
4: Lactate dehydrogenase in *P. fennica* — a trophozoite
5: α-glycerophosphate dehydrogenase in *P. fennica*, *a* — a trophozoite, *b* — flagellate forms after palintomic division
6: Glutamate dehydrogenase in *P. fennica* — flagellate forms
7: β-hydroxybutyrate dehydrogenase in *P. fennica*, *a* — a trophozoite, *b*, *c* — flagellate forms
8: Succinate dehydrogenase in *P. macrogranulata*, *a* — a trophozoite immediately after its removal from the host intestine, *b* — a predivision trophozoite, which has stayed in water for some time, *c* — a flagellate form
9: Malate dehydrogenase in *P. macrogranulata*, *a–c* — trophozoites
10: Isocitrate dehydrogenase in *P. macrogranulata*, *a*, *b* — trophozoites
11: Lactate dehydrogenase in *P. macrogranulata*, *a*, *b* — trophozoites
12: α-glycerophosphate dehydrogenase — a trophozoite
13: β-hydroxybutyrate dehydrogenase in flagellate form of *P. macrogranulata*
14: Glutamate dehydrogenase in *P. macrogranulata*, *a*, *b* — flagellate forms

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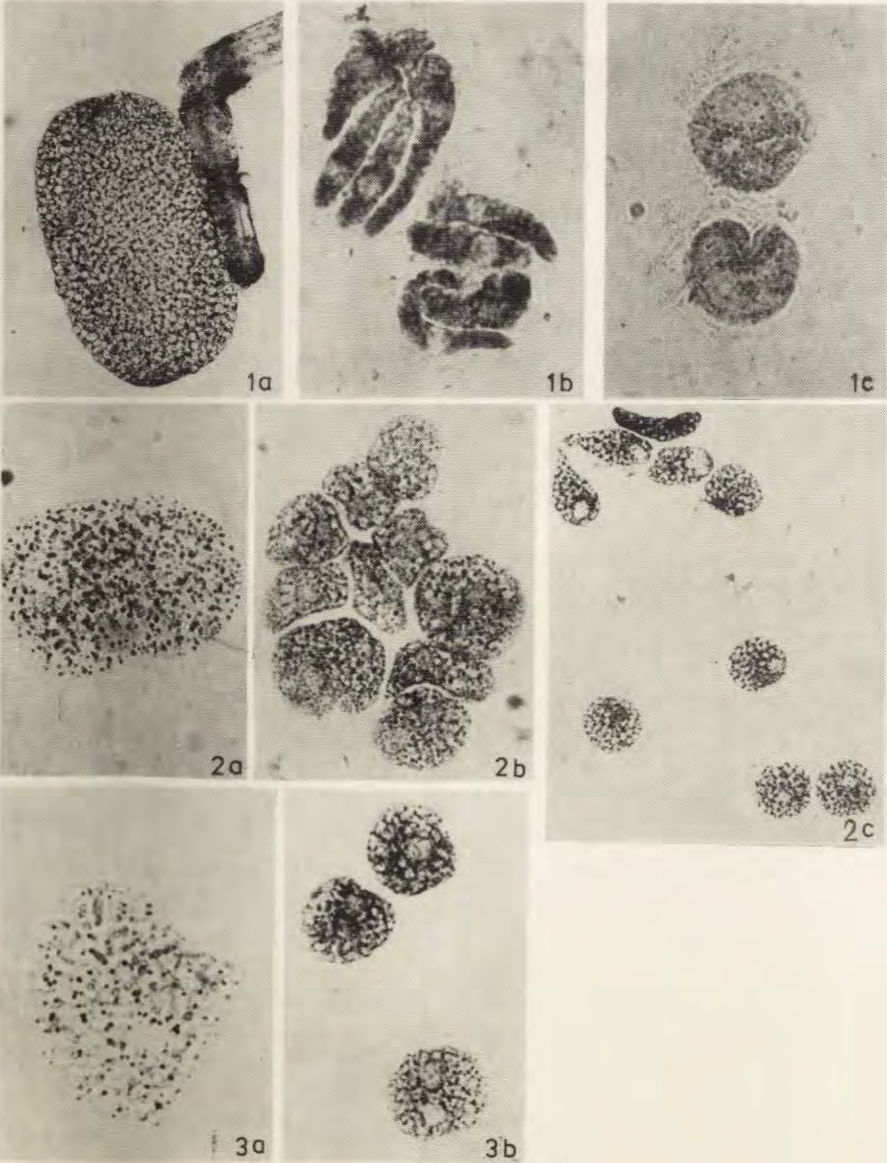

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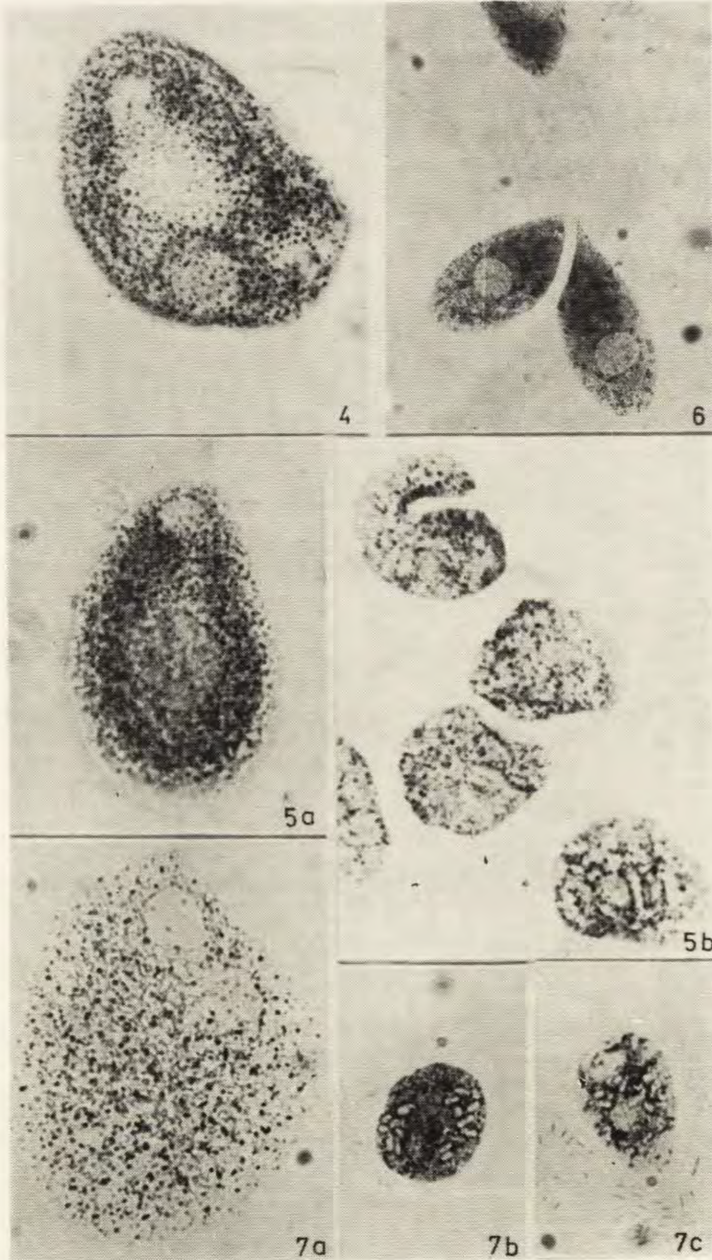

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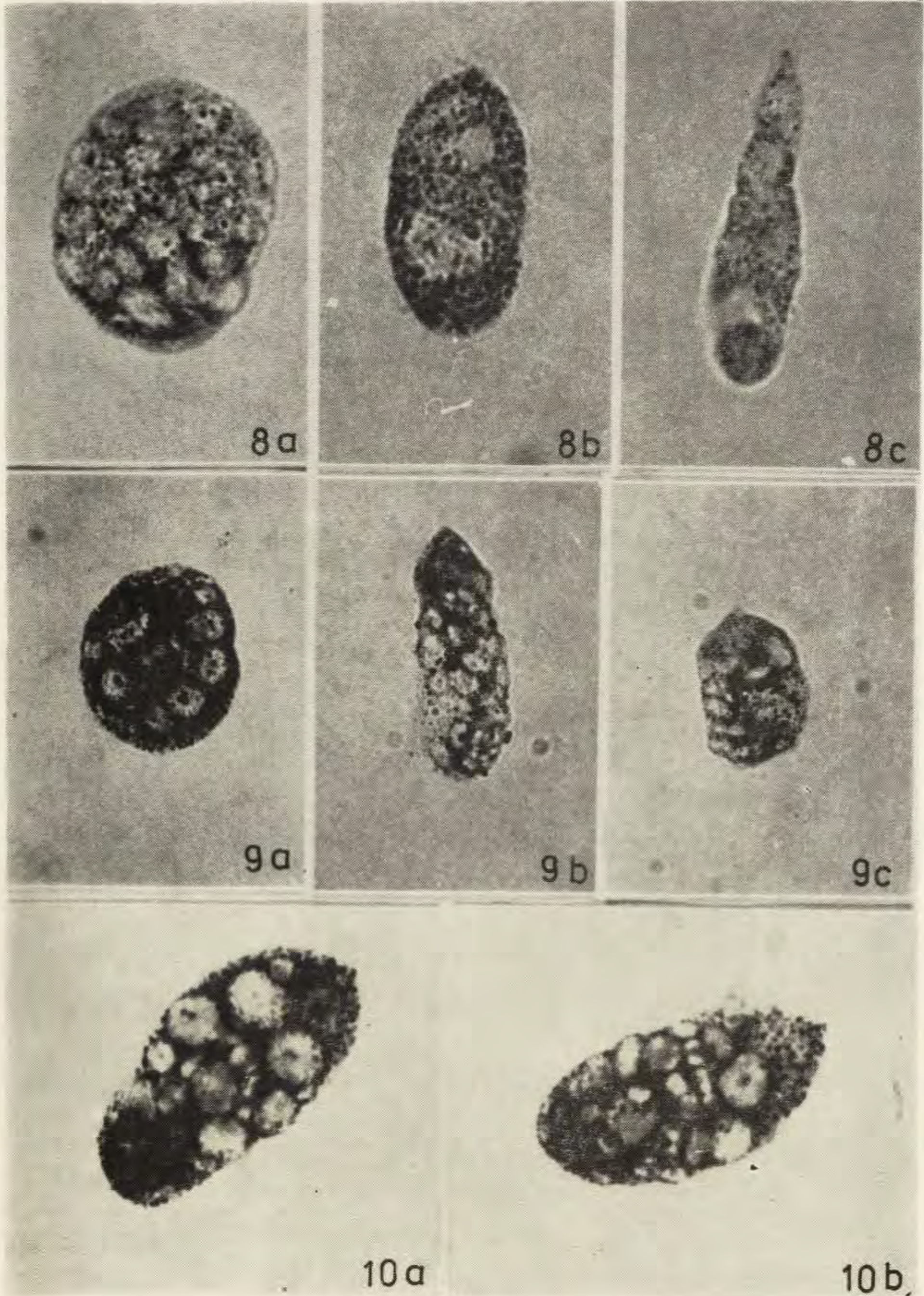

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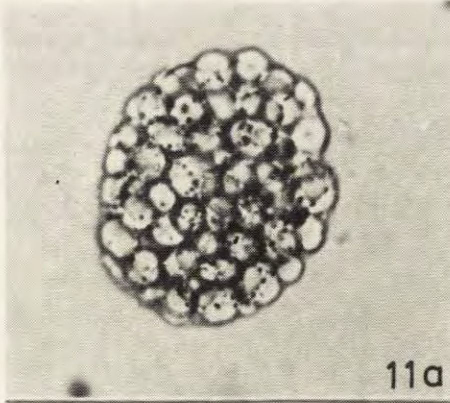

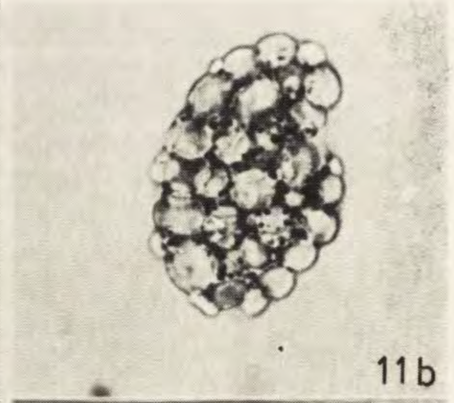

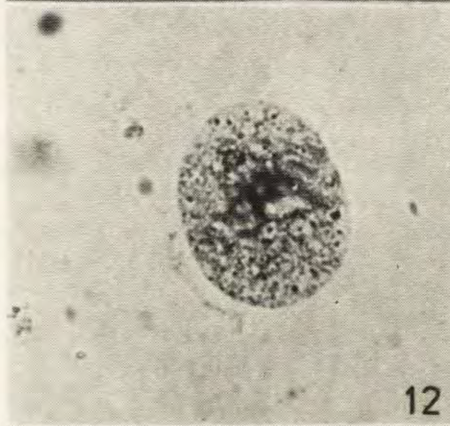

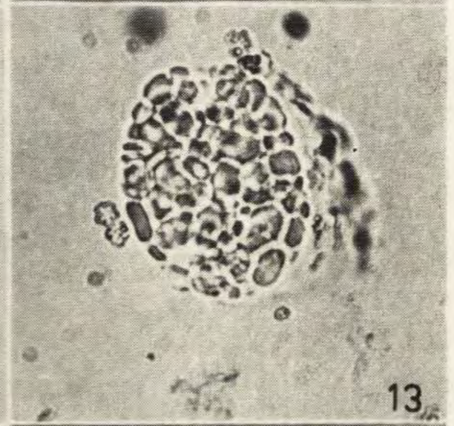

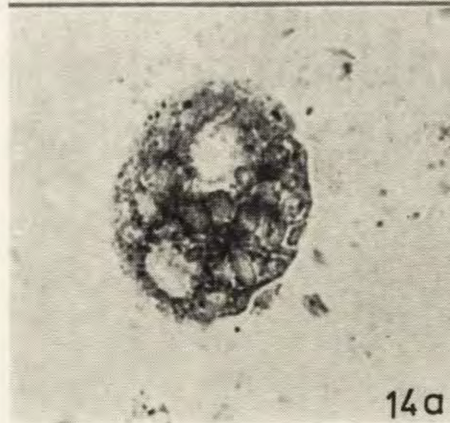

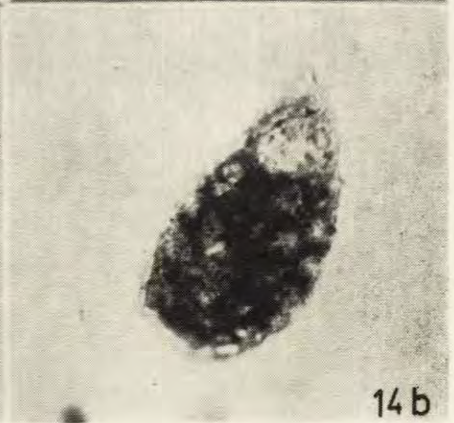

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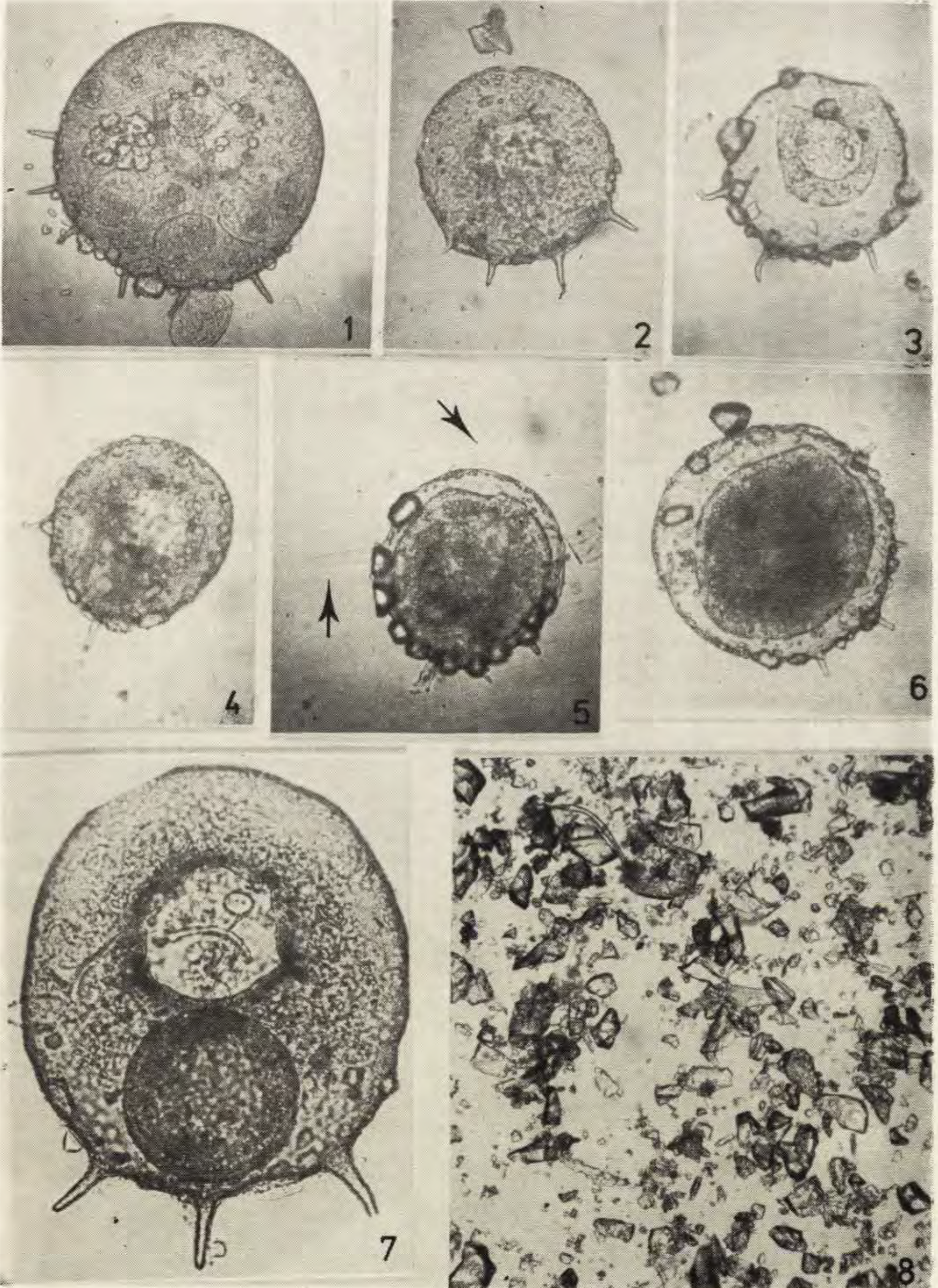

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ACTA PROTOZOOLOGICA
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Sur la multiplication de *Centropyxis discoides* et l'influence du milieu sur la morphologie de la thèque (*Rhizopoda testacea*)

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Synopsis. Nous décrivons une expérience d'élevage de *Centropyxis discoides* dans un milieu mixte, afin de contrôler le peuplement obtenu sans renouvellement du milieu.

Cette expérience, permet également de noter les variations intraspécifiques dans un milieu restreint, proche des conditions naturelles. Nous obtenons outre une bonne prolifération pendant les quinze premiers jours, une réduction sensible de la taille et une disposition anachronique des cornes. Les premières manifestations d'enkystement, sont apparues au 16° jour.

Chez les Thecamoebiens, au cours des générations, la morphologie de la thèque s'organise progressivement en fonction du milieu qu'ils colonisent. Cette faculté, se démontre expérimentalement par des élevages en milieux conditionés.

Dans cette expérience, nous avons utilisé *Centropyxis discoides* (Penard) Deflandre; espèce aquatique, commune également parmi les Mousses humides et les Sphaignes.

La structure de la thèque est principalement constituée d'un vernis organique souvent de teinte verte, généralement incrusté de particules minérales, de plaquettes siliceuses polymorphes ou de Diatomées remaniées ou non.

Matériel et méthode

Deux *Centropyxis discoides* typiques, provenant d'un dépôt lacustre, ont été mis en élevage dans un milieu mixte, constitué d'une solution de Ringer additionnée de quelques gouttes d'eau d'origine, de quelques milligrammes de Protenum

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Neutre (Chardez 1986) et de micromorpholithes de silice: ces fines particules minérales, sont obtenues en broyant du sable marin au mortier, puis traité par l'acide nitrique à chaud pour éliminer toutes matières organiques, ensuite, lavé soigneusement par l'eau distillée à plusieurs reprises.

L'élevage est réalisé en boite de Petri de 9 cm de diamètre à la température ambiante et sous éclairage naturel.

Sans autre apport, des Bactéries et des Ciliés amenés par l'eau d'origine, peuvent également se développer dans ce milieu.

Seule l'évaporation est compensée régulièrement par de l'eau distillée.

Les caractéristiques des deux *C. discoides* implantés sont les suivantes:

(1) Diamètre de la thèque 210 μm, nombre de cornes 6

(2) Diamètre de la thèque 148 μm, nombre de cornes 5

La thèque des deux spécimens était incrustée de fines particules minérales et de quelques Diatomées remaniées.

Résultats

Sans renouvellement du milieu, l'expérience a duré 20 jours, et a donné un développement maximum de 409 espèces actives, avant les premiers enkystements apparus au 16° jour; au 18° jour, de nombreuses thèques étaient soit: vides, soit: envahies par des Bactéries.

Le dénombrement exacte devenant impossible, on peut dire que cet élevage a donné un rendement en flèche du 8° au 15° jour (Fig. 1).

En même temps que les *Centropyxis*, se sont développés des Ciliés des Genres *Paramecium*, *Chilodonella*, *Vorticella* et un Rotifère du Genre *Monostyla*.

Chez les *Centropyxis*, les modifications les plus importantes résident dans une réduction de la taille de la thèque et du nombre de cornes; sur 100 individus pris au hazard, nous comptons 30 spécimens possédant de 5 à 6 cornes, 58 de 1 à 4 cornes, ces cornes sont disposées irrégulièrement et souvent assez courtes ou tordues, enfin, 12 spécimens sans corne, elles sont souvent remplacées par des éléments pierreux (Pl. I 5).

La teinte des thèques, ne subit pas de grands changements, habituellement plus claires au début de l'élevage elle fonce au bout de quelques jours.

Le nombre de premières formes prékystiques, apparues après 16 jours, augmente assez rapidement en raison de l'appauvrissement du mileu.

C'est à ce moment, qu'il convient de renouveler le milieu si l'élevage doit être continué. Les Thecamoebiens adherent sur le fond de la boite de Pétri donc, il suffit de verser lentement le liquide surnageant et de le remplacer par une solution conforme à celle du départ.

Chez les Thecamoebiens, la forme prékystique qui précède normale-

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ment la formation du kyste véritable (Pl. I 7) peut durer longtemps, tant que la disette persiste, c'est une attente de conditions nutritionnelles plus favorables.

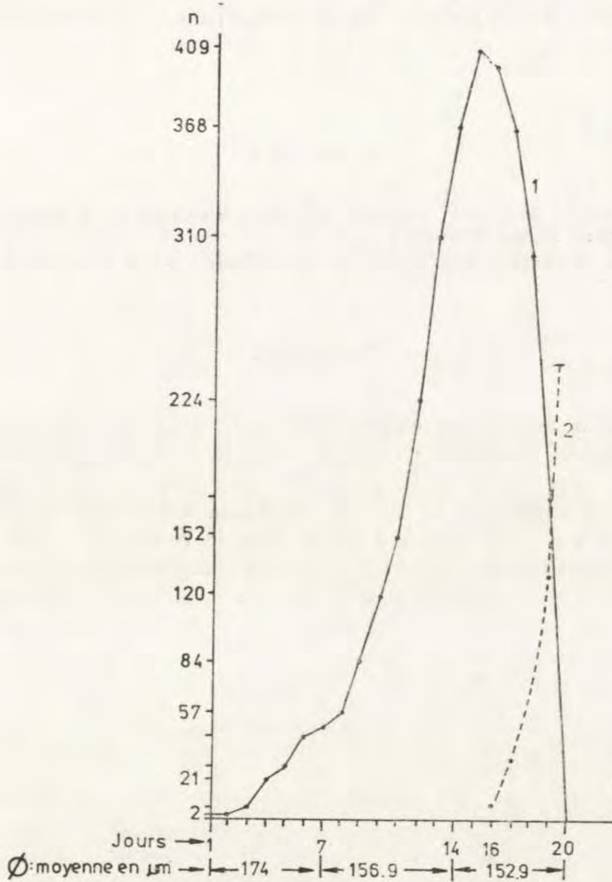

Fig. 1. *1* — Développement des Centropyxis, *2* — Apparition des Prékystes

Ce cas est habituel chez les espèces aquatiques adaptées à des milieux soumis à des périodes de sécheresse et d'humidité alternatives, telles que les Mousses, ou les Sphaignes sur sol.

Conclusion

Ce type d'expérience, permet de mettre en évidence des phénomènes de microendémicités chez certaines espèces vivant en milieux restreints, phénomènes qui se passent couramment dans la nature et expliquent cer-

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taines variations entre populations de même espèces, vivant dans les grandes pièces d'eau et les petites mares de la plaine.

Ces modifications se rencontrent en particulier chez les espèces dont la thèque est consolidée par des matériaux d'origine exogènes, la morphologie générale de la thèque est naturellement conditionée par les matériaux présents dans le milieu.

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SUMMARY

We describe a cultivation experiment of *Centropyxis discoides* in a mixed medium, in order to control the number of individuals obtained without medium renovation. This experiment also allows the observation of intraspecific variations in a restrained medium close to natural conditions. We obtain, beyond a good proliferation during the first fifteen days, a sensible reduction of the test size and an anachronic disposition of the korns. The first manifestations of encysting appeared on the 16th day.

EXPLICATION DE PLANCHE I

1: Spécimen implanté à 6 cornes
2 a 7: Descendants possédent 4, 3, 2 et sans cornes (les flèches indiquent les pseudopodes)
6: Prékyste (× 200)
7: Kyste de résistance (× 400)
8: Micromorpholithes de silice, obtenus par broyage de sable marin (× 600)

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Morphology and Life-cycle of *Retractocephalus melanopli* sp. n. (*Didymophyidae, Eugregarinida*) from the Gut of *Melanoplus* sp. (*Orthoptera*)

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Synopsis. The morphology and life-cycle of a new gregarine, of the order *Eugregarinida*, *Retractocephalus melanopli* sp. n. from the midgut of an orthopterous insect *Melanoplus* sp. is described. The early stages of the development of the parasite are intracellular. Epimerite: 12.0–24.0 μm hyaline, button-like with a small depression could be retracted into a cup-like depression in the protomerite. Sporonts: 132.0–576.0 × 34.0–180.0 μm with a subspherical protomerite. Sporadins: biassociative. Primite: globular and small. Cysts: spherical 208.0–310.0 μm in diameter. Gametes: isogamous, oval, 8.0 × 4.0 μm. Spores: barrel-shaped with thin wall, 12.8 × 5.6 μm. Dehiscence by simple rupture.

Haldar and Chakraborty (1976) established a new genus *Retractocephalus* to accommodate a parasite from the midgut of *Rhaphidopalpa* (=*Aulacophora fabecollis*). They characterized the genus by the presence of an epimerite which can be retracted into the protomerite. Since then five species, *R. aulacophora*, *R. spatulatus*, *R. spinosus*, *R. halicus* Haldar and Chakraborty, 1982 and *R. waltairensis* Rajakumari and Narasimhamurti, 1984 were added to the genus. All the species described so far are from coleopterous insects.

The present parasite is from the gut of *Melanoplus sp.* (*Orthoptera*) and differs considerably form all the species described so far. For reasons discussed later in this paper it is considered new to Science.

Material and Methods

Melanoplus sp. collected from different areas in and around the University Campus at Waltair (Andhra Pradesh, India) during the winter months (November–January) were brought to the laboratory decapitated and examined for gregarine

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parasites. Smears prepared from the infected insects were fixed either in hot Schaudinn's fluid or in Carnoy's fluid and stained using routine histological techniques such as Heidenhain's iron haematoxylin, Ehrlich's acid haematoxylin, Delafield's haematoxylin or treated according to the Feulgen's technique. Gametocysts were collected from the hindgut and were kept in 2.5% aqueous potassium

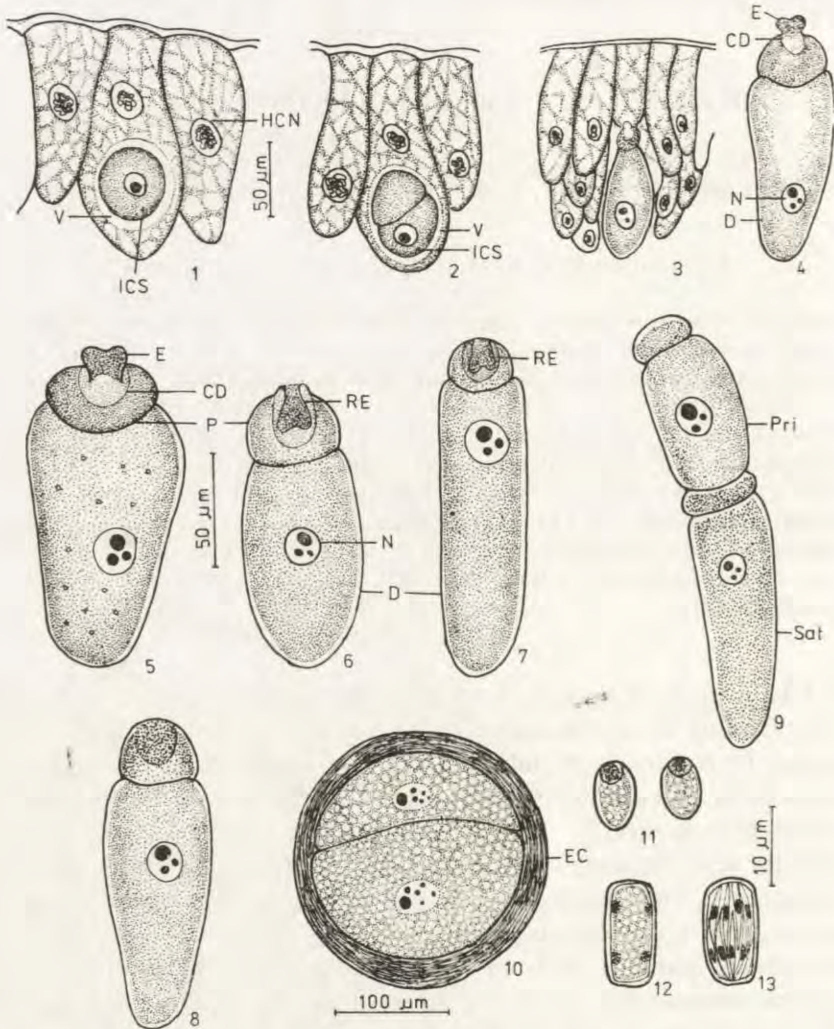

Fig. 1. *Retractocephalus melanopli* sp. n. *1–2* — Endogenous stages in the midgut epithelial cell, *3* — Young cephalont attached to the epithelial cells, *4* — Cephalont with fully extended epimerite, *5–7* — Mature cephalonts with retracted epimerite, *8* — Sporont, *9* — Association stage, *10* — Gametocyst, *11* — Gametes, *12* — Tetranucleate spore, *13* — Spore with sporozoites

Abbreviations: C, D—Cup-like depression, D — Deutomerite, E — Epimerite, HCN — Host cell nucleus, ICS — Intracellular stage, N — Nucleus, P — Protomerite, Pri — Primite, RE — Retracted epimerite, Sat — Satellite, V — Vacuole

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dichromate at 25–28°C to observe gametogenesis and sporogony. For studying the endogenous stages, guts of the infected hosts were fixed in the Bouin–Duboscq fluid, sectioned at 8 μm thickness and stained suitably.

Observations

Retractocephalus melanopli sp. n.

Host: *Melanoplus* sp.
Site of infection: Midgut
Locality: Waltair, Andhra Pradesh (India)
Type slides: Authors' collections and Department of Zoology, Andhra University, Waltair

Eighty to ninety per cent of adult *Melanoplus* sp. collected during November-January period were infected with this parasite.

Endogenous stages observed in the epithelial cells of the midgut were spherical measuring 22.0-48.0 μm in diameter with a vesicular nucleus containing a eccentric endosome. Parasites were always found in vacuoles (Fig. 1 *1*). With the growth of the parasite the body showed a demarcation into two segments, presumably representing the protomerite and deutomerite. The segmented stages were oblong or oval and they measured 52.0-75.0×24.5-32.6 μm with two equal segments and a clear septum dividing them. The nucleus was situated in one of the two segments (Fig. 1 *2*).

Young cephalonts attached to the epithelial cells of the midgut measured 60.5-94.5×32.5-56.5 μm. The epimerite was simple, globular and button-like with a small depression in the centre and it measured 10.0-12.0×12.0-24.0 μm. The protomerite was rectangular with rounded corners and it measured 18.0-38.0×32.0-52.0 μm. A lightly stained hyaline area was present in the protomerite just below the epimerite which appeared like a hollow cup-like depression. In this region the septum was indistinct (Fig. 1 *3* and *4*). Various stages of sinking of the epimerite into the depression of the protomerite were observed in smears (Fig. 1 *5-7*). The cytoplasm was deeply stained. The variety of deutomerite shapes was observed: subspherical, oblong, elongated or cylindrical. The nucleus was vesicular with a distinct nuclear membrane and two or three karyosomes. The cytoplasm was finely granular. Mature cephalonts were fonud free in the lumen of the midgut and they measured 96.0-160.0×44.0-72.0 μm. They exhibited characteristic curling movements (Table 1, 2).

Sporonts measured 132.0-576.0×34.0-180.0 μm. They had a subspherical protomerite and an elongated deutomerite. The hyaline cup-like area present in the cephalont was not clear, but it appeared more deeply

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Table 1
Retractocephalus melanopli sp. n. Body measurements of cephalonts and sporonts (in microns)

	Cephalonts		Sporonts	
	Range	Mean	Range	Mean
Total length of body (TL)	60.5–160.0	121.25	132.0–576.0	354.12
Width of body (WL)	32.5–72.0	52.56	34.0–180.0	152.25
Length of protomerite (LP)	18.0–38.0	23.00	24.0–96.0	60.00
Length of deutomerite (LD)	72.0–128.0	102.10	96.0–480.0	288.96
Width of protomerite (WP)	32.0–52.0	42.00	30.0–192.0	110.00
Width of deutomerite (WD)	44.0–72.0	58.50	34.0–180.0	
Size of epimerite	8.0–12.0 × 12.0–24.0			
DL: TL	1: 1.5		1: 5.23	
LP: TL	1: 8		1: 6.8	
WP: WD	1: 1.5		1: 1.833	
LP: LD	1: 6.4		1: 4.2	

stained than the rest of the cytoplasm. Hence it was presumed that when the cephalonts separated from the epithelial cell the epimerite instead of getting detached, got retracted gradually into the hyaline cup and was finally absorbed into the cytoplasm (Fig. 1 7 and 8).

Table 2
Retractocephalus melanopli sp. n. Comparative body measurements of primite and satellite

	LP: TL	LD: TL	WP: WD
Primite	1: 10.5	1: 2.863	1: 1.9
Satellite	1: 18.0	1: 2.36	1: 2.117

Sporadins were biassociative. Association was caudo-frontal. Primite was always globular and smaller than the satellite (Fig. 1 9).

Cysts were spherical having a diameter of 208.0-310.0 μm and they passed out at a very early stage of development. The ectocyst was 25.0 μm thick (Fig. 1 10). Gamete formation was completed in 36-38 h after the cysts passed out. They were isogamous and oval (8.0×4.0 μm) with an anteriorly placed vesicular nucleus and a deeply stained endo-

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Table 3

A detailed comparison of the characters of the species of *Retractocephalus* described so far

Characters	*R. raphidopalpae*	*R. aulacophorae*	*R. spatulatus*	*R. spinosus*	*R. halticus*	*R. waltairensis*	*R. melanopli* sp. n.
Total length of cephalont	87.5–360.0 μm	40–410 μm	120–220 μm	100–300 μm	210–440 μm	21–137.77 μm	60.5–160 μm
Epimerite	Simple globular	7.5–15.0 μm simple globular	5.0–12.5 μm simple globular	10 μm in length knob-like	10–20 μm oval	9×7 μm knob-like, globular	10×16 μm simple, button-like with a small depression
Deutomerite	Cylindrical with distinct epicyteal striations	Obese: distinct epicyteal striations	Obese: distinct epicyteal striations	Cylindrical: distinct epicyteal striations only in sporadins	Cylindrical No epicyteal striations	Long Cylindrical or oval	Subspherical, oblong, elongated or cylindrical No epicyteal striations
Nucleus	Spherical or oval, 1–3 karyosomes	Spherical, 1–2 karyosomes	Oval, 1 or 2 karyosomes	Spherical, 1 and in rare cases 2 karyosomes	Spherical, 1 or 2 karyosomes	Spherical, 1 endosome	Spherical, 2 or 3 karyosomes
Sporadins	Biassociative	Biassociative	Biassociative	Biassociative	Biassociative	Biassociative	Biassociative
Gametocyst	Oval, 250×210 μm gametocytes equal: dehiscence about 48 h after	Spherical 150 μm, gametocytes equal: dehiscence about 22 h	Oval, 150×130 μm, gametocytes equal: dehiscence about 24 h	Spherical or slightly oval 120 μm in dia. to 200×162 μm gametocytes equal: dehiscence about 48 h	Spherical, 260–280 μm gametocytes equal: dehiscence about 48 h	—	Spherical, 208–310 μm, gametocytes unequal: dehiscence 90 h
Spore	7×4 μm barrel-shaped	10×4 μm barrel-shaped	7×4 μm barrel-shaped	8×4 μm barrel-shaped	9×4 μm barrel-shaped	—	10.8×5.6 μm barrel-shaped
LP:TL	1:4.9	1:4.8	1:5.0	1:5.7	1:72	1:10.6	1:5.23
WP: WD	1:1.2	1:1.3	1:1.5	1:1.5	1:2.4	1:2.5	1:1.8
Site of infection	—	—	—	—	—	Malpighian tubules and haemocoel	Midgut
Host	*Raphidopalpa (Aulcophora) foveicolli* Lucus	*Aulacophora intermedia* Jaeoby	*Lema* sp.	*Monolepta signator* Oliv	*Haltica* sp.	*Rhapidopalpa stevensi*	*Melanoplus* sp.

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some (Fig. 1 *11*). Sporogenesis was completed in 84-90 h. Dehiscence was by simple rupture. Spores were barrel-shaped with a thin wall and they measured 12.8×5.6 μm. They were octozoic (Fig. 1 *12* and *13*).

Systematic Position

The new parasite is included in the genus *Retractocephalus* Haldar and Chakraborty, 1976 because of the presence of a hyaline knob-like epimerite which can be completely retracted into a depression in the protomerite and biassociative sporadins. A comparison of the cephalonts and sporonts with the species already described showed that it comes closest to *R. rhapidopalpae* and *R. halticus* measuring 87.5-360.0 μm and 210.0-440.0 μm respectively. The cephalonts of the new species are bigger than those of all the previously described ones. The epimerite in the new form is hyaline, button-like with a small depression, unlike in the other species described so far where it is simple and globular. A detailed comparison of the species discribed is given in Table 3.

Because of the morphological differences of the devlopmental stages and since this parasite is the first case of *Retractocephalus* from an orthopterous insect, *Melanoplus* sp., it is considered a new species for which the name *Retractocephalus melanopli* is proposed, after its host.

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Gregarina nymphaeae sp. n., a New Eugregarine Parasite of *Galerucella nymphaeae* L. (*Coleoptera: Chrysomelidae*)

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Synopsis. Adults of *Galerucella nymphaeae* L. collected in August 1987 on *Nymphaea alba* L. in Lake Monate close to Varese (Northern Italy), were infected by eugregarine *Gregarina nymphaeae* sp. n. The gregarine inhabits the intestine, and its sporonts form syzygies of two individuals. The maximum length of sporonts being in syzygies is 250 μm while of solitary sporonts ready for syzygies is 205 μm. The ratio of length of protomerite to total length (LP:TL) of primite sporonts ranges between 1:3.1 and 1:5.6. The taxonomic position of *Gregarina nymphaeae* sp. n. and *Gregarina munieri* (Schneider) known to parasitize in several chrysomelid species is discussed.

A leaf beetle *Galerucella* (*Hydrogaleruca*) *nymphaeae* L. is a common insect feeding on leaves of an aquatic plants *Nymphaea* spp. and *Nuphar* spp. Geus (1969) lists *G. nymphaeae* among big group of hosts for parasitic eugregarine *Gregarina munieri* (Schneider). In this paper we describe a newly recorded gregarine from *G. nymphaeae* with differs morphologically from *G. munieri* in so many respects that it justifies describing it as a new species named *Gregarina nymphaeae* sp. n.

Material and Methods

The adults and larvae of *Galerucella nymphaeae* were collected in September, 1987 on *Nymphaea alba* L. growing in Lake Monate close to Varese (Northern Italy). Insects were dissected and the content of their gut was examined microscopically at the magnification of 100× to 400×. Observed gregarines were measured and photographed in a saline solution at dark field or at normal light illumination.

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The holotype slide is deposited in the collection of the Institute of Plant Protection in Poznań; the paratype slides are deposited in the Istituto Entomologia Agraria in Bari.

Key to abbreviations used in tables and in the text is as follows: TL — total length of cephalont or sporont; LP — length of protomerite; LD — length of deutomerite; WP — width of protomerite; WD — width of deutomerite.

Results

Morphology

Cephalonts. The youngest cephalonts observed are from 48 to 61 μm long. The body is divided by septa into the epimerite, protomerite, and deutomerite (Pl. I 1,2). The epimerite is globular and up to 6.8 μm in diameter (Pl. I 2).

The protomerite of the cephalont is elongated and narrowed in front, and this shape is frequently preserved in young sporonts (Pl. I 3). Septum and constriction is well seen. Endocyte is granular.

The deutomerite is elongated and narrows slightly at the posterior end. The ectoplasm is thin and the endocyte is moderately granular and partly translucent.

The ratio of length of protomerite to total length (LP:TL) is 1 : 3.45 and varies from 1 : 3.2 to 1 : 3.9 (Table 1).

Sporonts solitary. Young sporonts being solitary differ from sporonts being in syzygies, and are from 109 to 205 μm long (Table 1). The protomemrite of solitary sporont frequently preserves the feature of protomerite of cephalont as it is elongated and sometimes club-shaped (Pl. I 3,4). There are, however, also solitary sporonts with hemispherical protomerite (Pl. I 5).

The deutomerite of solitary sporont is elongated with posterior end truncated. The ectocyte is thin and the endocyte has fine granulation and is partly translucent (Pl. I 3-5). The nucleus is at the posterior end of deutomerite, occasionally in the middle, and contains a single karyosome.

The ratio of length of protomerite to total length (LP : TL) is 1 : 4.45 and varies from 1 : 3.8 to 1 : 5.5 (Table 1).

Sporonts in syzygies. Sporonts being in syzygies differ morphologically from solitary sporonts. Although primites are slightly smaller than satellites their morphological features are very much alike (Pl. II 6,7), The length of primites varies from 99 to 223 μm and of satellites from 115 to 250 μm (Table 1).

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Table 1

Measurements of cephalonts and sporonts of *Gregarina nymphaeae* sp. n. from *Galerucella nymphaeae* L.

	TL (μm)	LP (μm)	LD (μm)	WP (μm)	WD (μm)	LP:TL	WP:WD	WP:LP
Cephalonts (n = 10)								
Range	45–61	14–17	31–44	14–17	17–31	1:3.2–3.9	1:1.0–1.8	0.82–1.0
Mean	53.4	15.5	37.9	16.4	22.0	1:3.45	1:1.33	1:0.94
Sporonts[1] (n = 10)								
Range	109–205	26–45	90–163	26–42	41–90	1:3.8–5.5	1:1.2–2.3	1:0.68–1.15
Mean	150.8	33.9	116.8	32.5	58.6	1:4.45	1:1.8	1:0.96
Sporont [2] (n = 10)								
Primite: Range	99–223	22–51	70–182	16–48	22–85	1:3.1–5.6	1:1.2–2.1	1:0.68–1.10
Mean	168.8	38.3	129.8	32.4	55.5	1:4.4	1:1.7	1:1.18
Satellite: Range	115–250	22–45	93–208	22–68	42–102	1:4.1–6.5	1:1.0–1.9	1:0.68–1.10
Mean	172.9	35.1	137.8	44.0	64.9	1:4.9	1:1.47	1:0.80

[1] solitary

[2] in syzygies

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The protomerite of primites and satellites is ovoidal, longer than wide, with slight constriction and well seen septum (Pl. II 6, 7). Ectoplasm is very thin and endoplasm is moderately granular, having the same granulation as the deutomerite what is well seen in the dark field illumination (Pl. II 6). Plasmolysis effect is seen in case of water mount or prolonged mounting in saline solution (Pl. II 7).

The deutomerite of primite and satellite is cylindrical, and narrows slightly towards the posterior end. The ectoplasm is thin and the endoplasm is moderately granular.

The nucleus is at the posterior end of the deutomerite and contains a single karyosome (Pl. II 6).

The nucleus has a diameter of about 21 μm, and the karyosome of about 12 μm.

In case of primite the ratio of length of protomerite to total length (LP: TL) is 1 : 4.4 and varies from 1 : 3.8 to 1 : 5.5. In case of satellite the ratio LP: TL is 1 : 4.9 and varies from 1 : 4.1 to 1 : 6.5 (Table 1).

C y s t s. In insects examined in August 1987 practically only cephalonts and sporonts were observed. However a lew non-matured cysts (Pl. III 8) having diameter from 210 × 120 to 280 × 160 μm were observed in three host insects.

Infection Level and Intensity

Infection is prevalent among larvae and pupae as the parasite was present in 98% of examined larvae. In case of adult insects the infection level decreases to 31%. This indicates that infected larvae die or that during pupation period some insects are able to escape from infection due to removal of parasitic gregarines with disintegrated larval tissues.

The intensity of infection, that is the number of parasites inside the gut of host insects, particularly in larvae, is very high (Pl. III 9, 10) which evidently causes some harm to them.

Taxonomic Position

Although no data on sporoducts are available since only non-matured cysts were observed but the morphological features of the cephalonts and sporonts indicate that this gregarine belongs to the genus *Gregarina*.

G e u s (1969) lists *Galerucella nymphaeae* among 79 species of *Chrysomelidae* family as host for *Gregarina munieri* (S c h n e i d e r 1875). In fact *G. munieri* is quite polyphagous as indicated by studies of T h e o d o r i d e s and J o l i v e t (1959), L i p a (1967) and L i p a and S i m c h u k (1979).

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However, the gregarine recorded in *Galerucella nymphaeae* and studied by us cannot be identified as *G. munieri* due to clearly different morphological features and due to fact that it forms syzygies of only two individuals while *G. munieri* frequently forms syzygies with two satellites (Lipa and Simchuk 1979, Geus 1969).

As seen in Table 2 such features like length and width of primitesporont, ratios of LP : TL and WP : LP, shape of protomerite and deutomerite and location of nucleus in deutomerite strongly indicate that gregarine recorded in *Galerucella nymphaeae* is a new species for which we propose a name *Gregarina nymphaeae* sp. n.

Discussion

Lipa and Simchuk (1979) reporting infection of *Chrysomela menthastri* (Suffr.) by *Gregarina munieri* (Schneider) and *G. crenata* (Bhatia et Setna) emphasized that gregarine infections among chrysomelids were recorded in different parts of the world. However, some authors believe that these infections are caused only by one or two gregarine species. Theodorides (1954) synonimized *Gregarina diabrotica* Kamm-Watson (1918) described in North America with *Gregarina munieri* Schneider (1875) described from other hosts in Europe.

Geus (1969) listed *G. munieri* as parasite of 79 species of *Chrysomelidae* family. In fact *G. munieri* seems to have a world-wide distribution and to be quite polyphagous. Lipa (1967) recorded it in *Chrysomela coerulans* Scriba while Lipa and Simchuk (1979) found it in *Chrysomela menthastri* Suffr. However, the oppinion expressed by Theodorides and Jolivet (1959) that *G. munieri* has host insects in families *Chrysomelidae, Clytriidae, Cassidae* and *Curculionidae* has not been confirmed and creates some doubts.

It is doubtful to consider that species belonging to such a big family like *Chrysomelidae*, having world-wide distribution feeding on so many plant species and inhabiting so many biotopes would be parasitized only by one gregarine species, namely *Gregarina munieri*. Although Geus (1969) listed only *G. munieri* and *Steinina ellipsoidalis* Zwetkow from 79 chrysomelid species Lipa and Simchuk (1970) proved that *Chrysomela menthastri* is parasitized by *Gregarina munieri* and *Gregarina crenata* Bhatia et Setna (1924).

Lipa and Simchuk (1979) emphasized that there is a lack of data on variations in the size, morphology and life cycle of *G. munieri* developing in various hosts occurring in different geographical regions. Such a study would be of great value as it would provide information on

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Table 2

Comparison of mean measurements of primite-sporonts of *Gregarina nymphaeae* sp. n. and *Gregarina munieri* (Schneider) and their characteristics

Features of primite	*Gregarina nymphaeae* sp. n. Host: *Galerucella nymphaeae* L. Ref.: this paper	*Gregarina munieri* (Schneider)		
		Host: not given Ref.: Geus (1969)	Host: *Chrysomela coerulans* Scriba Ref.: Lipa (1967)	Host: *Chrysomela menthastri* Suffr. Ref.: Lipa and Simchuk (1979)
Length (mean)	168.8	623.7	421.2	314.7
Width (mean)	55.5	216.5	193.2	101.5
LP:TL (mean)	1:4.4	1:4.77	1:5.66	1:5.70
WP: WD (mean)	1:1.70	1:1.52	1:1.74	1:1.60
WP:LP (mean)	1:1.18	1:0.91	1:0.71	1:0.62
Shape of protomerite	Conical	Hemispherical	Hemispherical	Hemispherical
Shape of deutomerite	Cylindrical	Elongated	Elipsoidal	Cylindrical and ovoidal
Location of nucleus in deutomerite	At posterior end	At posterior end or in the middle	At anterior end	At anterior end
Number of Karyosomes	1	1	1	1

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biotypes and varieties of *G. munieri* which probably exist due to the great number of suspected hosts and the broad distribution.

As seen in Table 2 there is a quite big variation in mean length and mean width of primite-sporonts of *G. munieri* reported by Geus (1969), Lipa (1967), Lipa and Simchuk (1979) and Theodorides and Jolivet (1959). However, such features like shape of protomerite and deutomerite, location of nucleus in deutomerite and ratios LP : TL, WP : WD and LP : TL indicate that in fact this is *G. munieri* mentioned by the above authors.

However, as seen in Table 2 the size parameters and morphological features of *G. nymphaeae* sp. n. recorded in *Galerucella nymphaeae* L. so greatly differ from size and morphological features of *G. munieri* that this justifies to consider it as a new species named *G. nymphaeae* sp. n.

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EXPLANATION OF PLATES I–III

1–2: Young cephalonts of *Gregarine nymphaeae* sp. n. with body divided to epimerite (E), protomerite (P), and deutomerite (D)

3–5: Solitary sporonts of *G. nymphaeae* sp. n. showing some variations in the shape of protomerite and deutomerite

6: Sporont of *G. nymphaeae* sp. n. in syzygies photographed at dark field microscopic illumination, a nucleus, with single karyosome, located at the posterior end of deutomerites is well seen

7: Sporonts of *G. nymphaeae* sp. n. in syzygy, photographed at normal microscopic illumination; a plasmolysis process is observed condensing endocyte in central parts of deutomerites

8: Unmatured cyst of *G. nymphaeae* sp. n. in intestine of adult host insect

9–10: Groups of gamonts and sporonts of *G. nymphaeae* sp. n. in squashed gut tissues of *G. nymphaeae*

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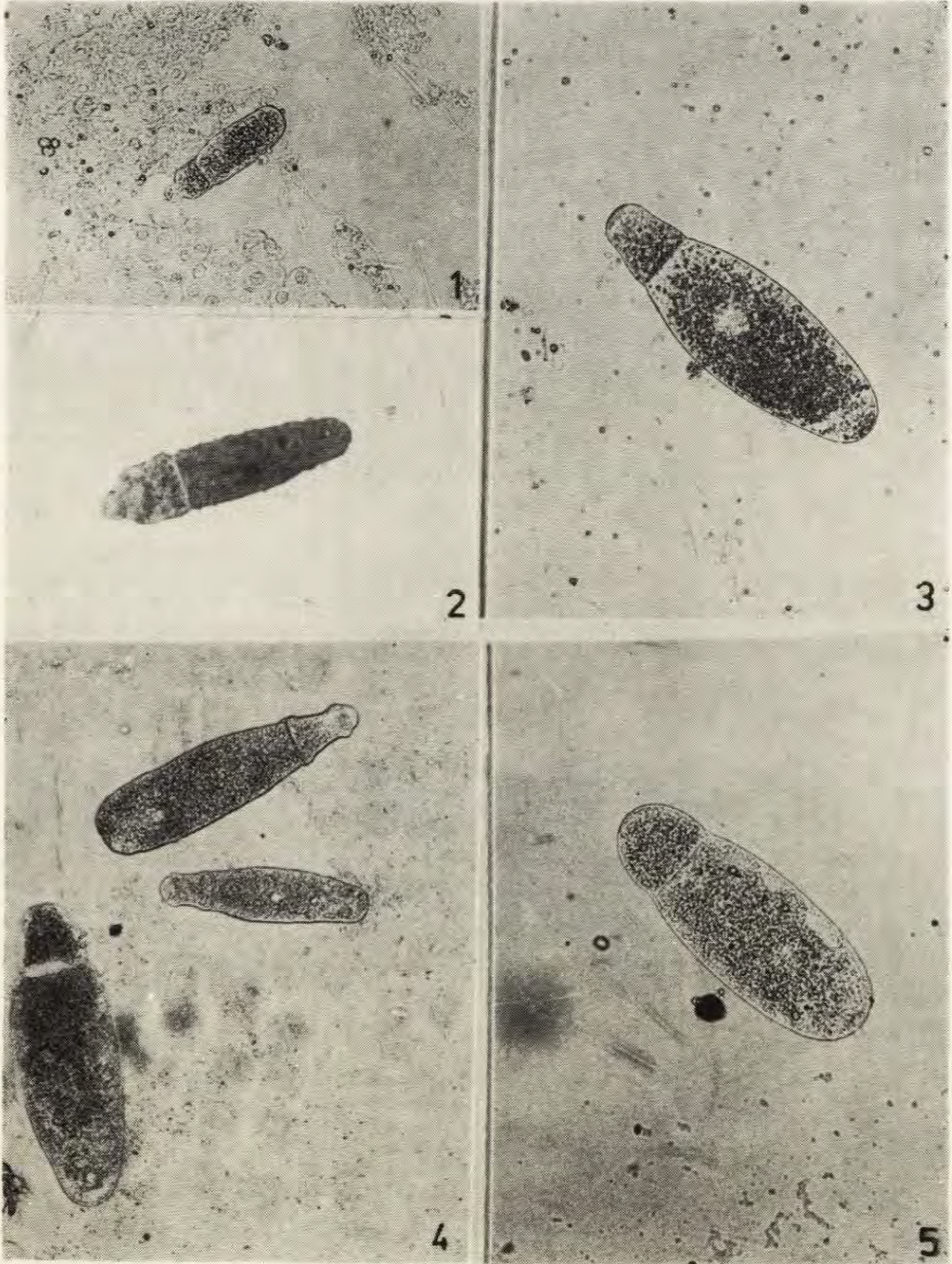

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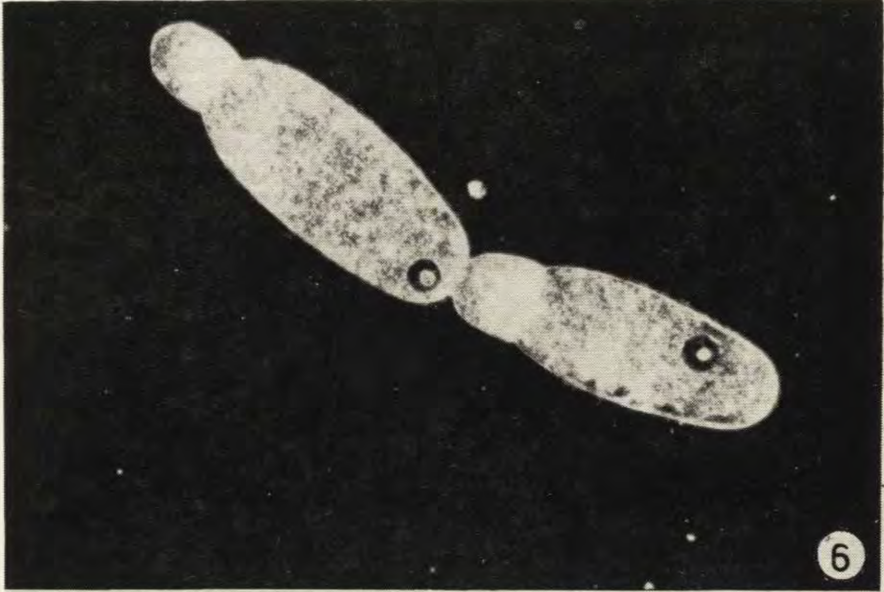

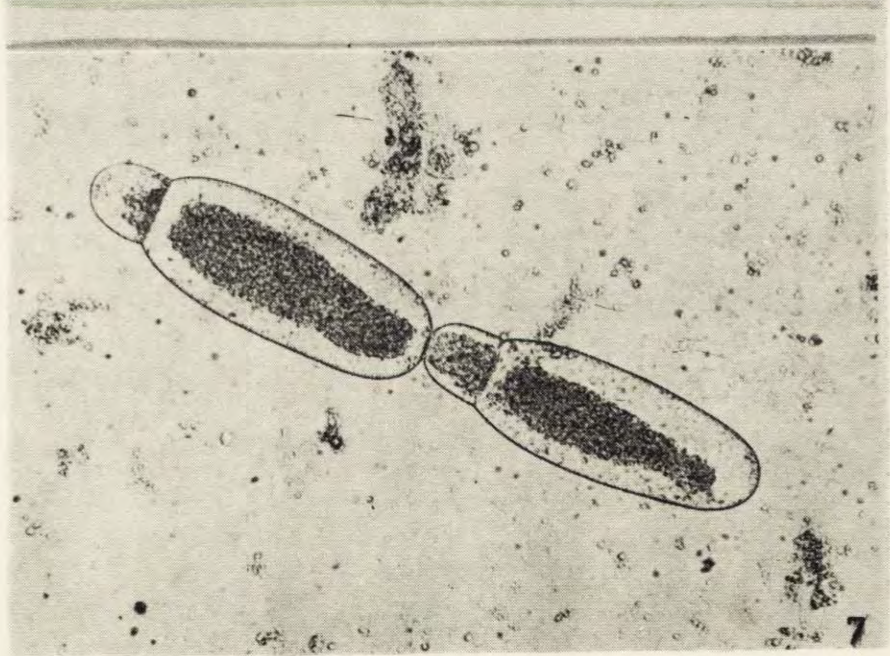

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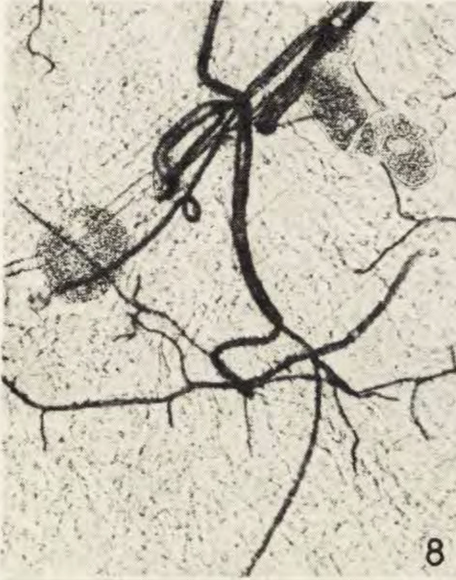

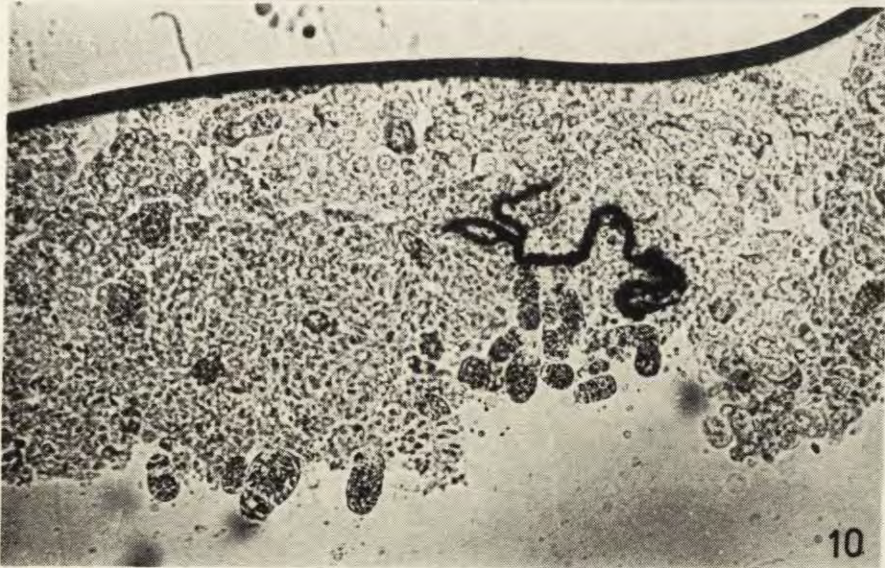

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Role of Environmental Factors in the Incidence of Two New Species of Apicomplexan Parasites, *Hirmocystis lophocateri* sp. n. and *Hirmocystis triboli* sp. n. from Coleopteran Insects

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Synopsis. The paper deals with the role of environmental factors on the incidence of infection of *Hirmocystis lophocateri* sp. n. and *Hirmocystis triboli* sp. n. from *Lophocateres pusillus* (K.) and *Tribolium castaneum* (Herbst) respectively. The structure and affinities of the gregarines are also given in this paper.

Eugregarines found as parasites in the gut of insects are know to cause extensive damage in the host gut epithelia during their early development. Literature on their morphology and life cycle is extensive. Watson (1916), Lipa (1967), Haldar et al. (1986) and others have suggested that these parasites may be used for the biological control of harmful insect pests. However, it was felt necessary that the successful use of eugregarines as possible agents for the biological control of insect pests will be possible only when the nature of their incidence is known in detail. The present paper incorporates the role of environmental factors on the incidence of two new species of eugregarines (*Apicomplexa* : *Sporozoea*) belonging to the genus *Hirmocystis* Labbé.

Materials and Methods

Insect hosts were collected from spices and nuts at grocers' shops in the locality those were exposed to almost the same normal outside temperatures and humidity. These were brought alive to the laboratory for examination. The hosts were examined systematically for studying the seasonal intensity in the incidence of infection. Various meteorological data like temperature and humidity were main-

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tained to determine the correlation between the factors and incidence of infection of the parasites in the hosts. The data were plotted in composite curves. For studying parasites' structures, slides were stained with iron-alum haematoxylin and for the development of the gametocyst the technique of Sprague (1941) with minor modification was adopted.

Table 1

Showing the monthwise data of number of hosts examined, number of hosts parasitized, percentage of infection, average temperature and average humidity with reference to *Hirmocystis lophocateri* sp. n. in *Lophocateres pusillus* (K.) (adults) infesting *Arachis hypogea* L.

Month	Number of hosts examined	Number of hosts parasitized	Percentage of infection	Average temperature (°C)	Average humidity (%)
1983					
June	25	6	26.0	34.3	80.1
July	30	11	36.0	31.1	88.8
August	32	15	49.0	31.0	85.9
September	49	15	32.0	31.5	82.7
October	61	12	20.0	31.5	79.5
November	72	Nil	Nil	23.9	72.8
December	28	Nil	Nil	22.5	71.5
1984					
January	49	Nil	Nil	23.7	75.8
February	27	Nil	Nil	24.0	69.3
March	54	Nil	Nil	25.9	68.8
April	29	Nil	Nil	30.2	68.5
May	58	10	18.0	33.0	70.7
June	32	8	25.0	35.6	80.5
July	31	14	45.0	36.5	80.2
August	26	14	54.0	33.5	84.7
September	17	7	41.0	32.5	75.2
October	13	2	15.0	26.0	73.5
November	12	Nil	Nil	24.0	71.2
December	12	Nil	Nil	25.2	70.4
1985					
January	15	Nil	Nil	22.5	62.5
February	10	Nil	Nil	23.9	64.9
March	17	Nil	Nil	31.3	60.6
April	15	Nil	Nil	33.3	67.9
May	7	2	28.0	33.0	68.0
June	25	14	56.0	31.7	86.3
Total	746	130	17.42 (average)	—	—

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Observations

Factors Influencing Infection

The insect hosts *Lophocateres pusillus* (K.) and *Tribolium castaneum* (Herbst) have been systematically examined for gregarine parasites. In the case of *L. pusillus* first indication of infection was found in the month of June, 1983 and the examination of the hosts was then continued up to June, 1985. During this period a total of 746 hosts were examined, of

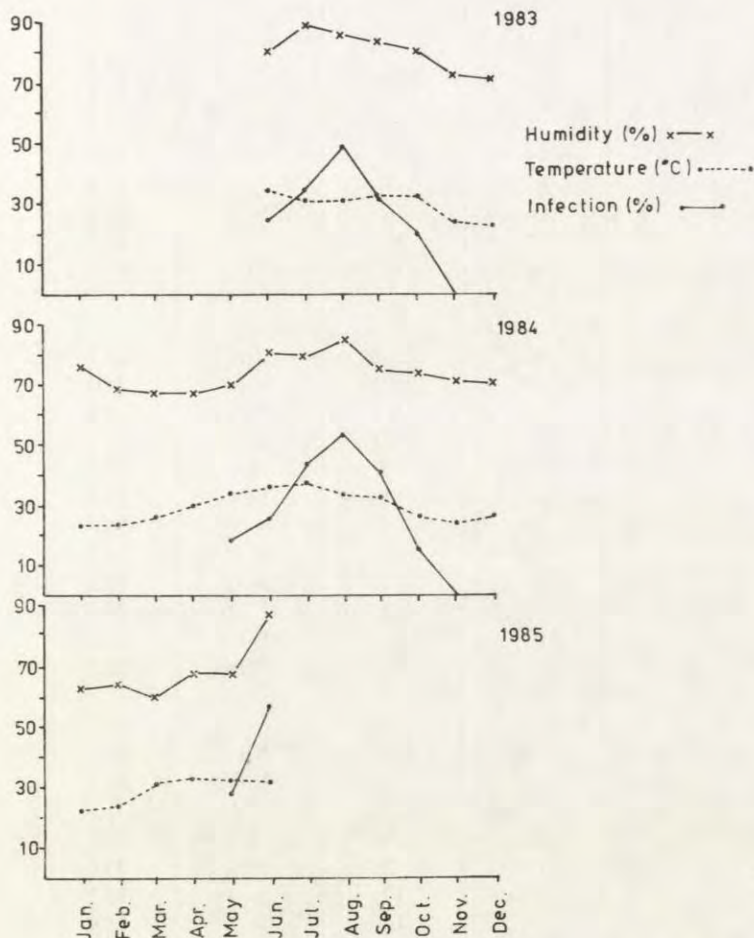

Fig. 1. Composite curve showing relation between environmental factors and infection of *H. lophocateri*
Abbreviations: Jan — January, Feb — February, Mar — March, Apr — April, May — May, Jun — June, Jul — July, Aug — August, Sep — September, Oct — October, Nov — November, Dec — December.

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which 130 were positive (Table 1). It has been noted that moderate to heavy infection is found during May to October in every year (1983–1985) after when it gradually declines to zero.

Table 2

Showing the monthwise data of number of hosts examined, number of hosts parasitized, percentage of infections, average temperature and average humidity with reference to *Hirmocystis triboli* sp. n. in *T. castaneum* (Herbst) (adult) in *Cuminum cyminum* L.

Month	Number of hosts examined	Number of hosts parasitized	Percentage of infection	Average temperature (°C)	Average humidity (%)
1983					
May	30	6	20.0	32.3	80.2
June	35	8	22.0	34.3	80.1
July	51	29	57.0	31.1	88.8
August	49	17	36.0	31.0	85.9
September	61	9	25.0	31.5	82.7
October	40	6	15.0	31.5	79.5
November	54	Nil	Nil	23.9	72.8
December	79	Nil	Nil	22.5	71.5
1984					
January	40	Nil	Nil	23.7	75.8
February	47	Nil	Nil	24.0	69.3
March	68	Nil	Nil	25.9	68.8
April	52	Nil	Nil	30.2	68.5
May	24	6	25.0	33.0	70.7
June	31	12	38.0	35.6	80.5
July	20	8	40.0	36.5	80.2
August	40	24	60.0	33.5	84.7
September	20	6	30.0	32.5	75.2
October	20	2	10.0	26.0	73.5
November	10	Nil	Nil	24.0	71.2
December	11	Nil	Nil	25.2	70.4
1985					
January	9	Nil	Nil	22.5	62.5
February	9	Nil	Nil	23.9	64.9
March	8	Nil	Nil	31.3	60.6
April	20	1	5.0	33.3	67.9
May	15	2	15.0	33.0	68.0
June	32	17	55.0	31.7	86.3
July	29	10	35.0	28.9	87.2
August	54	17	32.0	30.2	86.8
September	35	10	30.0	30.1	86.9
October	25	Nil	Nil	25.4	70.5
Total	1018	190	18.7 average	—	—

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It is very interesting to find that the incidence of infection has a direct relation with humidity and temperature. The high incidence in August, 1983 (49%), August, 1984 (54%) and June, 1985 (56%) is accompanied by a favourable daily average temperature of 31.0°C and humidity of 85.9% in 1983; 33.5°C and 84.7% in 1984; and 31.7°C and 86.3% in 1985 respectively. From the data recorded during the 25 months in course of this investigation it becomes clear that this particular parasite requires a specific range of humidity of 84.7% to 86.3% together with average daily temperature ranging from 31.0°C to 33.5°C for maximum

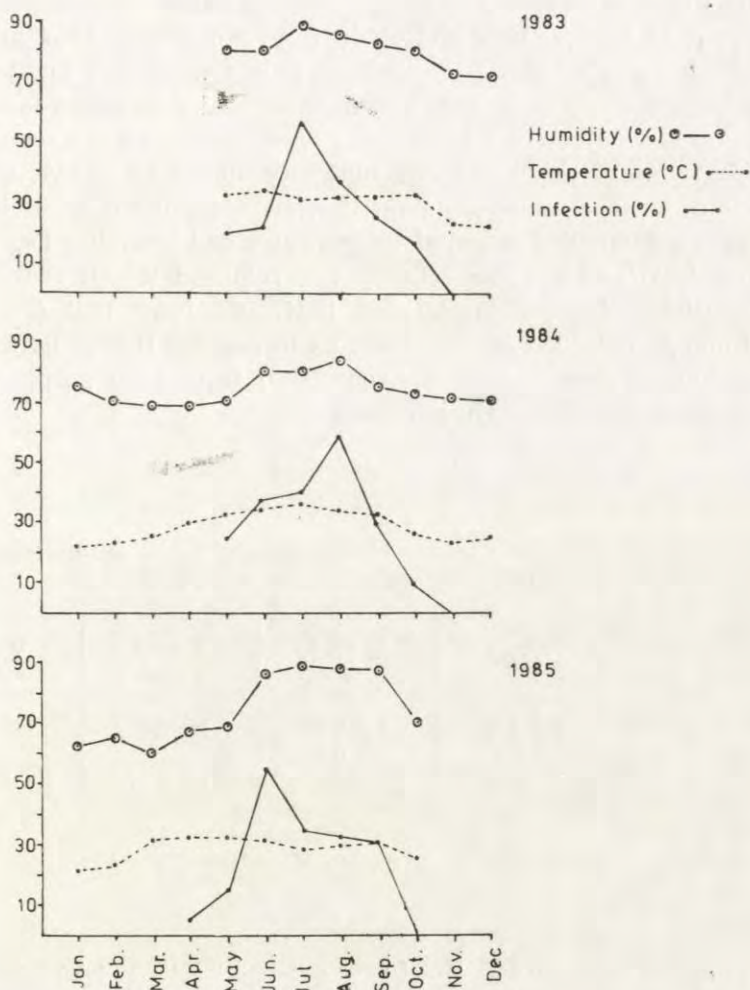

Fig. 2. Composite curve showing correlation between environmental factors and incidence of infection of *H. triboli*
For. abbreviations see Fig. 1.

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sporulation and propagation. It will be evident from Fig. 1 and Table 1 that variations in either of these factors adversely affect the incidence of infection in the host. For example, the percentage of humidity in July, 1983 is 88.8% and daily temperature is 31.1°C. Similarly in May, 1984 the average humidity is 70.7% while daily temperature is 33.0°C. In both the cases the incidence of infection is appreciably less than in August, 1984 and June, 1985, when both the factors are favourable for parasites to sporulate.

In case of *T. castaneum* the infection was noticed in May, 1983, where after the examination was continued up to October, 1985. During this period 190 out of 1018 hosts were found infected with gregarines (Table 2). As in the previous case the infection is altogether absent from November, 1983 to March, 1984 and again from November, 1984 to March, 1985, when the overall climatic condition is dry with very low temperature and humidity. As expected, moderate to heavy infection is obtained from May to September in all the years when average daily temperature ranges from 28.9°C to 36.5°C and humidity 70.7% to 88.8%, the most favourable conditions for sporulation; however, for the peak of infection in each year a particular range of temperature and humidity i.e., 31.1°C–33.5°C and 84.7%–88.8% respectively are required which are recorded in the months of July, 1983, August, 1984, and June, 1985 (Fig. 2).

The findings recorded above tempt us to suggest that individual gregarine species require specific environmental factors for successful sporulation and propagation in newer hosts.

Table 3
Showing the statistical relationship between the environmental factors and the incidence of infection in the two species of *Hirmocystis* Labbé

Name of the parasite	T vs. I P value	H vs. I P value	T vs. I t value	H vs. I t value
Hirmocystis lophocateri sp. n.	$r \geqslant 0.69$ $P < 0.01$	$r \geqslant 0.80$ $P < 0.001$	$t > 0.05$	$t > 0.001$
Hirmocystis triboli sp. n.	$r \geqslant 0.68$ $P < 0.01$	$r \geqslant 0.69$ $P < 0.01$	$t > 0.01$	$t > 0.001$

Abbreviations: T — Temperature, I — Infection, H — Humidity

The relationship between the rate of infection in the two cases and various environmental factors has been found statistically significant and has been depicted in Table 3.

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Structures and Affinities

Examinations of histological sections of infected host guts have revealed that both the gregarines undergo early intracellular development in the intestinal epithelia before becoming free in the host gut lumen. However, the gregarines exhibit certain features of their own which are described separately.

The gregarines (Fig. 3 *3-9*) parasitizing *L. pusillus* (K.) have the following features:

T r o p h o z o i t e. Solitary, elongated in shape with dome-shaped epimerite when fully expanded. The protomerite is short, pea-shaped and

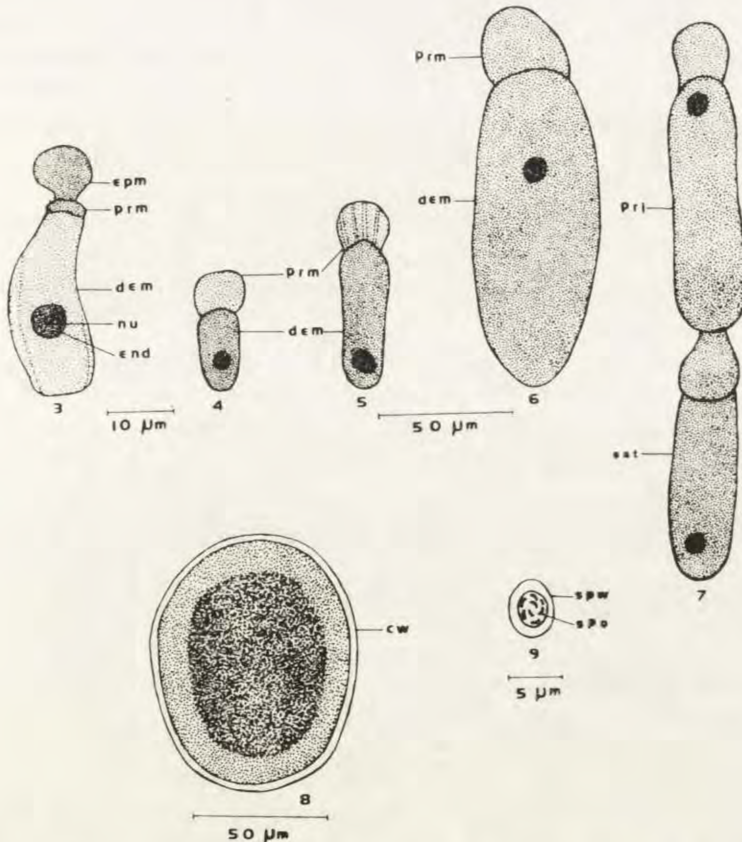

Fig. 3. Camera lucida drawings of the structural features of *H. lophocateri* sp. n. *3* — elongated trophozoite with dome-shaped epimerite, *4–6* — sporadins of various shapes and sizes, *7* — syzygy, *8* — ovoidal gametocyst, *9* — ovoidal spore *Abbreviations:* epm — epimerite, prm — protomerite, dem — deutomerite, nu — nucleus, pri — primite, sat — satellite, cw — cyst wall, spw — spore wall, spo — sporozoite, end — endosome.

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typically broader than long. A slightly concave septum separates the protomerite from the cylindrical deutomerite. The nucleus is rounded in shape with a clear endosome. Epicyteal striations are not observable.

Sporadin. Solitary as well as biassociative. Ovoidal to elongated in shape with hemispherical or tongue-shaped protomerite. The deutomerite is ovoidal to elongated in shape.

Association. Caudo-frontal. The primite and satellite are morphologically different. The primite is larger than the satellite and the protomerite of the primite is tougue-shaped or ovoidal whereas the protomerite of the satellite is more or less conical.

Gametocyst and spore. Blackish white in color and ovoidal in shape. The gametocyst measures 96.6 μm in length and 38.4 μm in width. The outer wall of the gametocyst is thicker. The central portion of the gametocyst is more condensed than the peripheral portion. The cyst dehisces by simple rupture at 40 h and liberates the spores in chains.

Double-walled, ovoidal spores measure 5.7 μm × 3.3 μm in the average. After 72 h of development inside the moist chamber eight rod-like sporozoites are developed in each spore (Biometry — Table 4).

Table 4

Showing number (n) examined, average ($\bar{x}$), standard deviation (s) and coefficient of variation (v) of *Hirmocystis lophocateri* sp. n. infecting *Lophocateres pusillus* (k)

	n	$\bar{x}$	s	v
Total length	35	56.7	30.45	53.7
Length of epimerite	5	7.2	2.07	28.75
Length of protomerite	35	16.7	5.43	32.51
Length of deutomerite	35	42.6	26.31	61.76
Length of nucleus	35	7.47	2.24	29.98
Width of epimerite	5	6.9	1.33	19.27
Width of protomerite	35	18.8	6.07	32.28
Width of deutomerite	35	22.1	9.36	42.35
Width of nucleus	35	8.09	3.02	37.33
Length of protomerite/ Total length	35	3.37	1.24	36.79
Width of protomerite/Width of deutomerite	35	0.94	0.33	35.1

Affinities. The frequent occurrence of biassociative sporadins, dehiscence of cyst by simple rupture and ovoidal spores at once place the parasite under the genus *Hirmocystis* Labbé. The parasite shows close similarites with *H. polymorpha* Labbé and *H. pseudoductis* Haldar and Chakraborty in having spherical nucleus, solitary as well as biassociative sporadins and ovoidal spores but differs in all other characters, i.e., shape

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of the epimerite, protomerite, deutomerite, gametocysts, ratios of LP : TL and WP : WD. However, LP : TL and WP : WD ratios are closely related with *H. theodoridesi* Kundu and Haldar. Its dome-shaped epimerite, pea-shaped deutomerite and ovoidal gametocysts differ from all other described species under the genus. It is, therefore, considered as a new species for which the name *Hirmocystis lophocateri* sp. n. is given.

Holotype: In slide No. LB/6, prepared on 12.7.1984.
Paratypes: In the above-mentioned slides and in other slides prepared on different dates.

The gregarines (Fig. 4 *10-17*) from *T. castaneum* (Herbst) have the following characters:

Trophozoite. Vase-like with tongue or papilla-like epimerite. The protomerite is dome or hat-shaped. The deutomerite is more or less globular in shape with a slightly tapering posterior end. The deutomerite

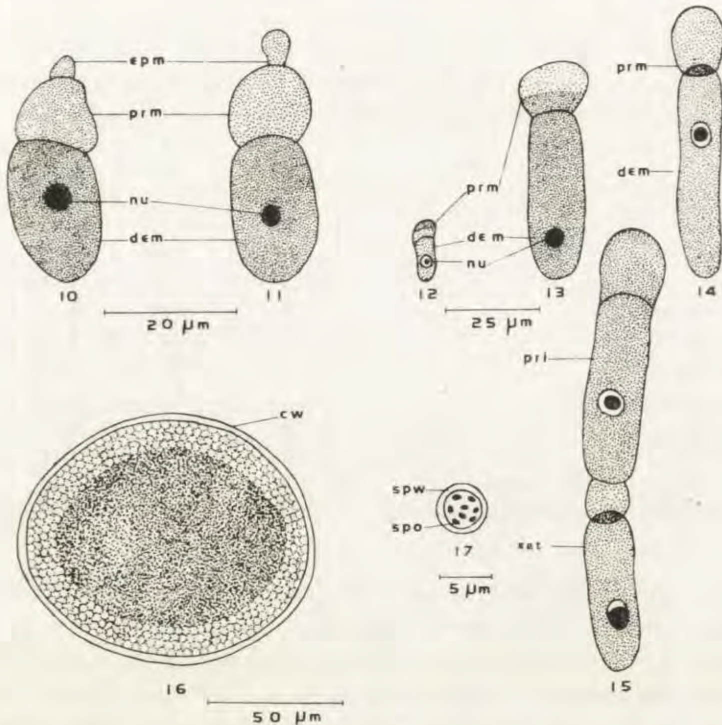

Fig. 4. Camera lucida drawings of the structural features of *H. triboli* sp. n., *10, 11* — fully grown trophozoites of different shapes and sizes, *12–14* — sporadins of various shapes and sizes, *15* — association, *16* — gametocyt, *17* — spore with eight sporozoites
Abbreviations: epm — epimerite, prm — protomerite, dem — deutomerite, nu — nucleus, pri — primite, sat — satellite, cw — cyst wall, spw — spore wall, spo — sporozoite.

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is separated from the protomerite by a slightly concave septum. The nucleus is spherical and situated at the middle region of the deutomerite.

Sporadin. Solitary or biassociative. Cylindrical in shape with hat- or spade-shaped protomerite. The cylindrical deutomerite encloses a rounded to spherical nucleus with prominent nuclear membrane.

Association. Caudo-frontal.

Gametocyst and spore. Whitish, double-walled spherical in shape measuring 119.7 µm × 96.6 µm. The central portion of the gametocyst is more condensed than the peripheral part. An ectocyst is present surrounding the cyst. The gap between the ectocyst and the gametocyst is about 23.1 µm. After 96 h of development the cyst bursts by simple rupture releasing the spores in masses.

Double-walled, rounded spores are 5.5 µm in average dimensions. After 30 h of development inside the moist chamber eight rounded sporozoites are developed in each spore (Biometry — Table 5).

Table 5
Showing the number (n) examined, average ($\bar{x}$), standard deviation (s) and coefficient of variation (v) of *Hirmocystis triboli* sp. n. infecting *Tribolium castaneum* (Herbst)

	n	$\bar{x}$	s	v
Total length	20	41.55	18.85	45.37
Length of epimerite	8	6.07	1.32	21.79
Length of protomerite	20	11.67	4.34	37.2
Length of deutomerite	20	27.44	16.36	59.63
Length of nucleus	20	6.06	1.67	27.68
Width of epimerite	8	5.7	0.85	15.07
Width of protomerite	20	13.22	5.19	39.3
Width of deutomerite	20	14.09	4.37	31.07
Width of nucleus	20	5.92	1.71	29.03
Length of protomerite/ Total length	20	3.48	0.43	12.63
Width of protomerite/ Width of deutomerite	20	1.11	0.29	26.63

Affinities. The gregarine has close similarities with *H. ventricossa* Labbé and *H. pitcharis* Haldar and Chakraborty ony in LP : TL ratios but differs from these two species in all other characters. The gregarine possesses tongue- or papilla-like epimerite and spherical gametocyst which are close to *H. asidae* Léger and *H. ventricossa* Labbé. However, these species differ widely in the LP : TL and WP : WD values. The gregarine also exhibits some resemblances with *H. oxeata* Ghose et al. in the shape of the sporadin, association and host species but differs in the shape and size of trophozoite, gametocyst, spore and LP : TL and WP : WD values.

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It is interesting to find that the same host insect (i.e., *Tribolium castaneum*) has been found to harbour two distinstly different species of parasites, viz., *H. oxeata* and the gregarine under report. However, the former has been obtained from hosts infesting walnuts (*Juglans regia* L.) while the presently described form has been collected from hosts infesting the common spicy fruits of *Cuminum cyminum* L. It has already been pointed out by Lipa (1967), Sengupta and Haldar (1986), Ghose et al. (1987) that the food habits of insects have some role in the distribution as well as morphological variations of gregarine fauna in their guts. The present investigation further confirms their contentions. All these features definitely justify that the gregarine from *T. castaneum* in the fruits of *Cuminum cyminum L.* is a new species which is named *Hirmocystis triboli* sp. n. after the generic name of its host.

Holotype: In slide No. JT/3, prepared on 2.5.1984.
Paratypes: In the above-mentioned slide and in other slides prepared on different dates.

Discussion

It is very clear from the present investigation that for the gregarine infection a particular range of temperature and humidity is required. The spores of the gregarines, as a rule, undergo development (i.e., sporulation) outside the hosts' gut. During high temperature in summer and low temperature in winter when there is minimum humidity, the spores are subjected to desiccation and hence the incidence of infection is at a low level in these months. During the rainy season, the average daily temperature ranges between 30.1°C to 36.5°C but there is sufficient humidity (80.1% to 88.8%) particularly in the Gangetic West Bengal, and therefore, the spores are subjected to least environmental hazards and sporulate normally. In a recent *in vitro* study Patil et al. (1985) have shown that temperatures of 30°C-40°C are optimum for sporocyst formation and maturity, while 35°C and below are most favourable for sporocyst viability. The present study also confirms thier results *in vivo*.

As the insect hosts as well as their gregarine parasites are very active during the months of June to September in a year, all measures for the biocontrol of the pests must be taken during this period. Since spore viability is very important for any effective programme, June–September may be considered most ideal and favourable for maintaining them *in vitro* and *vivo*.

As already pointed out by Ghose et al. (1986) the incidence of infection of gregarines may be considered an additional distinguishing

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feature for determining the taxonomic position of this group of parasites. This study also confirms their contention that a particular species of gregarines has a peak of incidence depending upon favourable conditions of temperature and humidity.

The slides containing the holotype and paratype materials of the two new species have been deposited in the Department of Zoology, University of Kalyani.

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A New Marine Psammobiotic Ciliate from the Japan Sea, *Trachelocerca obscura* sp. n. (*Ciliophora, Karyorelictida, Trachelocercidae*)

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Synopsis. A new species of the genus *Trachelocerca*, *T. obscura* sp. n., is described from medium-grain sand of the upper sublittoral of the Japan sea. The species is characterized by an elongated spindle-shaped body with a "head" and a pointed rear end, but without a tail-like extension, a length of 1.5–2 mm and a dark brown pigmentation. The number of kineties is 56 to 60; 7 to 14 macronuclei and 2 micronuclei are assembled into a loose group. A comparison is drawn with some previously known species in the genera *Trachelocerca* and *Tracheloraphis*.

The vast family *Trachelocercidae*, uniting almost exclusively obligately psammophilic marine ciliates, presently includes three genera (*Trachelocerca*, *Tracheloraphis* and *Trachelonema*) and at least 60 valid species, among them at least 40 species belonging to the genus *Tracheloraphis* (Dragesco 1960, 1963, 1965, Raikov 1962, 1963, Borror 1963, 1973, Agamaliev 1966a, b, Kovaleva 1966, Raikov and Kowaleva 1968, Kovaleva and Golemansky 1979, Wright 1982, and others). Species of this family play a leading role in many marine psammonic communities and, moreover, present a cytological interest. As all other representatives of the order *Karyorelictida*, they have macronuclei which are relatively poor in DNA (paradiploid) and unable to divide. Respectively, differentiation of some micronuclei into a new macronuclei occurs in the cycle of every cell division of these ciliates (review: Raikov 1985).

The form described below belongs to the genus *Trachelocerca* which is characterized by uniform ciliation, i.e., by the absence of a non-ciliated stripe or body side (Dragesco 1960).

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Material and Methods

Numerous individuals of the new species have been collected in August-September 1982 near the marine biological station "Vostok" of the Institute of Marine Biology (Vostok bight, Peter the Great gulf, Japan Sea). The ciliates were found in samples of oligosaprobic medium-grain sand taken under a water depth of 1.5–2 m in the First Priboynaia bay. They were extracted from sediment samples by the action of cold water obtained by thawing sea water ice in Uhlig's apparatuses (Uhlig 1964, 1968). The ciliates were studied *in vivo* and fixed (with simultaneous adhesion to slides) with Nissenbaum's (1953) fixative. The preparations were stained with Mayer's hemalum, methyl green and pyronin, or after Feulgen.

Description of *Trachelocerca obscura* sp. n.

Living stretched ciliates have a very elongate fusiform, almost unflattened body with a length-to-width ratio of about 20 : 1 (Fig. 1 a). The body length of such specimens is about 1.5–2 mm. The contractility is moderate: upon stimulation and during fixation the ciliates contract to about one-third, i.e., to 500–700 µm (Fig. 1 b). Living ciliates are conspicuously pigmented: with incident illumination, they appear dark brown, their coloration being so intense that it is comparable only with the pigmentation of *Geleia orbis* and by far exceeds that of any other trachelocercid (e.g., *Tracheloraphis dogieli* or *Tr. serratus*). With transmission illumination, their cytoplasm looks dark and hardly transparent (hence the proposed species name [1]).

The body smoothly narrows anteriorly into a flexible "neck" which carries an inflated apical "head". The rear end of the body is pointed but does not form a noticeable tail-like process (Fig. 1 a, b). The head is filled with refringent granules. There is no permanent mouth opening, the buccal zone being represented by a circular apical area of naked cytoplasm limited by only the plasma membrane [2] and surrounded by a peribuccal crown of cilia (Fig. 1 b).

The ciliature consists of 56–60 longitudinal kineties (in the middle part of the body). The non-ciliated (or glabrous) stripe is absent, which is a character of the genus *Trachelocerca*. However, there are an anterior and a posterior suture lines, which occur on one and the same ciliary meridian (Fig. 1 b). Up to 20 kineties, lying mainly to the right of this

[1] The best name for this species would have been the adjective "bruna", but that has already been used to designate one practically unidentifiable form (*nomen nudum*) of *Trachelocercidae* from the Black Sea, probably belonging to the genus *Trachelonema* (Lepşi, 1962).

[2] This has also been verified by electron microscopy.

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meridian, start from the anterior suture line and terminate against the posterior suture line (Fig. 1 b). The rest of kineties reach the anterior and the posterior body ends.

The brown body color depends on a multitude of pigment granules (protrichocysts), some 0.5 μm in diameter, which lie beneath the pellicle in the ridges between the ciliary rows (Fig. 1 c).

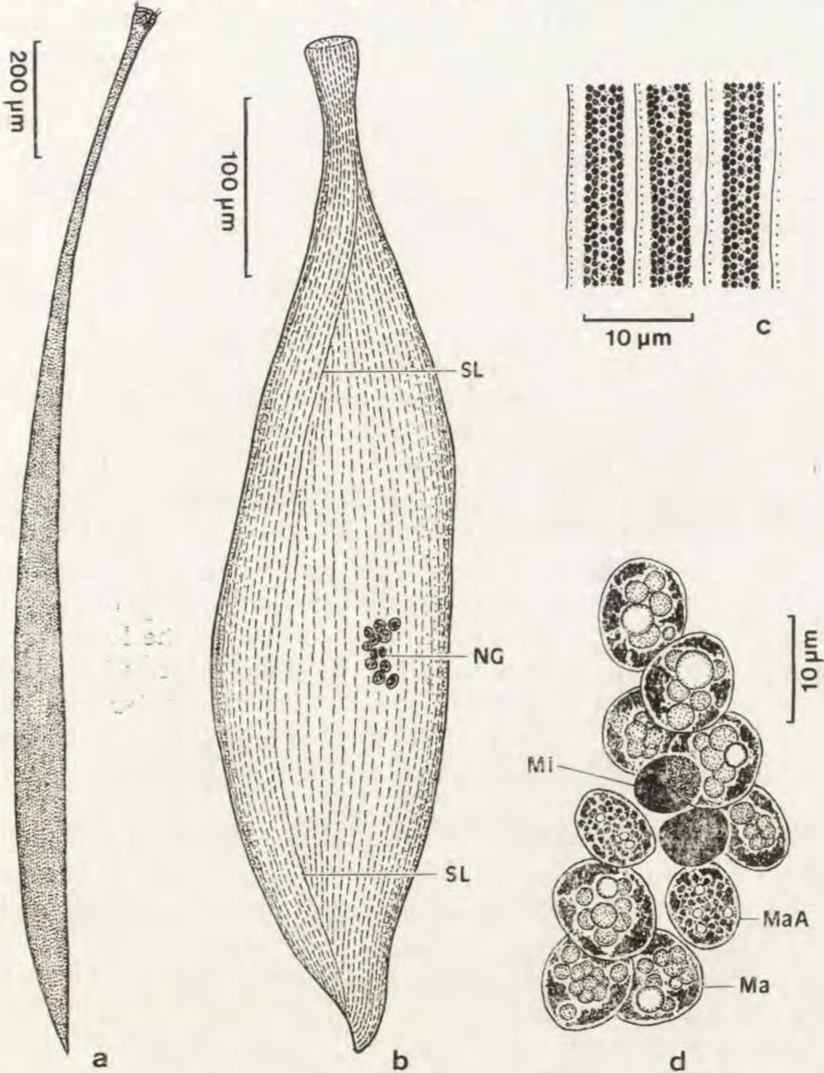

Fig. 1. Morphology of *Trachelocerca obscura* sp. n.: a — autline of a moving specimen, b— fixed specimen (hemalum staining) showing the suture lines (SL) and the nuclear group (NG), c — region of the cell surface with interkinetic pigment granules (*in vivo*); d — nuclear group (Feulgen — light green staining): Mi — micronuclei, Ma — macronuclei, MaA — macronuclear anlagen (developing macronuclei)

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The nuclear apparatus consists of a single rather loose group of nuclei located in the middle part of the body (Fig. 1 b). The group comprises 7 to 14 (most frequently 10) oval macronuclei, some 10 μm in size, and two rounded micronuclei measuring about 6 μm (Fig. 1 d). The macronuclei are placed rather randomly within the group and never form a compact nuclear complex.

Typical locomotion: sliding on a substratum. The thigmotaxis is very strong: stimulated and contracted ciliates firmly adhere to the surface of glass.

Biotop: medium-grain oligosaprobic sand of the upper sublittoral of the Japan Sea.

The type preparations are stored at the Laboratory of Cytology of Unicellular Organisms, Institute of Cytology of the USSR Academy of Sciences, Leningrad.

Discussion and Differential Diagnosis

The species described above needs to be compared with three other species of the genus *Trachelocerca* — *T. multinucleata* Dragesco, *T. geopetiti* Dragesco and *T. variabilis* Kovaleva (see Table 1). All these forms are large (more than 1 mm in length), more or less similar in body form, all have many (up to 60 or more) kineties. However, Table 1 shows that *T. obscura* differs from *T. multinucleata* by its larger body size and another composition of the nuclear apparatus; from *T. geopetiti*—by its pointed rear end, a higher number of macronuclei, and the looseness of the nuclear group; and from *T. variabilis* — by the presence of a "head" and another structure of the nuclear apparatus. The character most sharply distinguishing *T. obscura* from all the three species at once is, however, its conspicuous dark-brown body color depending on pigmentation of its protrichocysts.

It is useful to compare *T. obscura* also with those species of the neighbouring genus *Tracheloraphis* which are recorded to have a very narrow non-ciliated stripe (as narrow as the place occupied by 1 or 2 kineties). Though formally *T. obscura* clearly belongs to the genus *Trachelocerca*, the problem is that during fixation of some *Trachelocerca* species the suture lines tend to "open up" and then can be mistaken for exactly such narrow glabrous stripes. Therefore, included in Table 1 are also four species of *Tracheloraphis* showing very narrow non-ciliated stripes (*Tr. vermiformis*, *Tr. indistinctus*, *Tr. niveus*, and *Tr. lacteus*), and one species (*Tr. serratus*) with a somewhat wider glabrous stripe but, for that, with a brown pigmentation of the body. All five species have their nuclei gathered into a single group.

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Table 1

Differential diagnosis of *T. obscura* sp. n.

Genus and species	Front end	Rear end	Body length, mm	Body color	Protrichocysts	Number of kineties	Glabrous stripe	Nuclei (Ma/Mi)	Description(s)
Genus *Trachelocerca*:									
T. obscura sp. n.	Head with granules	Pointed without tail	1.5–2	Dark brown	Dark brown	56–60	Absent	7–14/2 Loose group	This paper
T. multinucleata Dragesco, 1960	No head	Pointed without tail	1.0–1.3	Colorless	Colorless	~60	Absent	20–24 Ma Long row	Dragesco 1960, 1963
T. geopetiti Dragesco, 1960	Head with granules	Rounded	1.0–1.8	Yellowish White	2 types, yellowish	50–60	Absent	4/2 Compact group	Dragesco 1960; Kovaleva and Golemansky 1979
T. variabilis Kovaleva, 1966	No head	Pointed without tail	0.8–1.8	White	Colorless	48–60	Absent	2–14/1–7 Short row	Kovaleva 1966, 1985
Genus *Tracheloraphis*:									
Tr. vermiformis Raikov, 1962	Head indistinct	Tail	~1.5	White	Colorless	55–70	Very narrow (2 kin.)	2–6/1–2 Group	Raikov 1962; Kovaleva 1967
Tr. indistinctus Wright, 1982	Head indistinct	Pointed without tail	0.6–1.2	Colorless	?	39–47	Very narrow (1 kin.)	4/2 Group	Wright 1982
Tr. niveus Wright, 1982	Head with granules	Rounded	0.6–1.5	White	?	36–47	Very narrow (1 kin.)	4/2 Loose group	Wright 1982
Tr. lacteus Raikov et Kovaleva, 1968	Large head with granules	Rounded	~1.5	White	Colorless	~40	Very narrow (1–2 kin.)	8/4 Group	Raikov and Kovaleva 1968
Tr. serratus Raikov et Kovaleva, 1968	No head	Pointed without tail	~1.5	Brown	Dark brown	40–45	Narrow (3–5 kin.)	8–12/2 Loose group	Raikov and Kovaleva 1968

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Table 1 shows that *T. obscura* cannot be identified with any of the above-named *Tracheloraphis* species even if one does not take into account the presence or absence of the glabrous stripe. In fact, *T. obscura* differs from *Tr. vermiformis* by the presence of a distinct "head", the lack of a tail-like process, and by the number of nuclei. From *Tr. indistinctus*, it differs by the existence of a "head", a higher number of kineties, and more numerous macronuclei; from *Tr. niveus* — by a pointed rear end and more numerous kineties and macronuclei; from *Tr. lacteus* — by a less inflated "head", pointed rear end and other numbers of both macronuclei and micronuclei. From all four species just mentioned, *T. obscura* clearly differs by the strong pigmentation of its body and protrichocysts. Finally, from *Tr. serratus*, a form with rather similar, though smaller nuclei, the species described here differs by the presence of a "head", more numerous kineties, and a darker brown color of the body. Moreover, the non-ciliated stripe is well expressed in *Tr. serratus* and it hardly can be an artifact of fixation, since pigmented protrichocysts are arranged onto it in a particular zig-zag fashion (this pattern is clearly seen *in vivo* as well).

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Ein neuer hypostomer Ciliat, *Pseudochlamydonella rheophila* sp. n. (*Pseudochlamydonellidae* fam. nov., *Pseudochlamydonella* gen. n.) im Aufwuchs eines Baches[1]

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Synopsis. *Pseudochlamydonella rheophila* sp. n., gen. n., fam. n., lebt im Aufwuchs von Quellen und Bächen des Sauerlands und Kottenforstes bei Bonn (Nordreihen–Westfalen, BRD). Die Art weist Bosonderheiten der Infraciliatur auf, die es rechtfertigen, ihr in der Unterordnung *Chlamydodontina* eine eigene Familie einzurichten. *Pseudochlamydonella rheophila* hat 1 Dorsalbürste, ventral 7 Kineten, davon 3 postorale. Die periphere Kinete liegt apikal. Kennzeichnende Familienmarkmale sind Kineten aus gepaarten, zick-zackförmig gestellten Kinetosomen sowie das Fehlen praeoraler- und circumoraler Kineten.

Nach Untersuchungen von Deroux (1970, 1976 a, b), Deroux und Dragesco (1968), Dragesco (1966), Foissner (1979), Heuss und Wilbert (1973), Pätsch (1974) und Wilbert (1971) sind hypostome Ciliaten in der Hauptsache Weidegänger, die sich von Bakterien und Algen ernähren. Ihr Habitat sind die verschiedenartigsten Substrate der Gewässer. Sie kommen im Süßwasser und besonders artenreich auch in marinen Lebensräumen vor. Hier soll nun ein sehr eigenaritiger Vertreter dieser Gruppe beschrieben werden, der im Aufwuchs von Fließgewässern lebt.

Material und Methode

Zur Gewinnung von Aufwuchs wurden in der Quellregion der Alme (Sauerland) und in Bächen des Kottenforstes (Bonn) Objektträger zur Besiedlung ausgebracht. Nach einer mehrwöchigen Expositionszeit wurde die gesamte Aufwuchsgesellschaft des Objektträgers nach der Methode von Wilbert (1976) präpariert.

[1] Gewidmet Herrn Prof. Dr. K. E. Wohlfarth-Bottermann zum 65 Geburstag.

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Ergebnisse

Ordnung: *Cyrtophorida* Fauré-Fremiet, 1956
Unterordnung: *Chlamydodontina*, Deroux, 1976
Familie: *Pseudochlamydonellidae* fam. n.

Familiendiagnose: Hypostome Ciliaten, deren Bewimperung ventral aus Dikineten besteht und dorsal bis auf die Dorsalbürste reduziert ist. Praeorale und circumorale Kineten fehlen. Makronucleus homogen, nicht in verschiedenartige Hälften zerlegt. Reusenapparat vom *Chilodonella*-Typ. Körper im Umriß breit-oval, ventral flach, dorsal gleichmäßig schwach gewölbt.

Typusgattung: *Pseudochlamydonella* gen. n.

Typuspräparat: Zoologisches Institut der Universität Bonn.

Gattungsdiagnose: Bogenförmig verlaufende Ventralkineten aus gepaarten Kinetosomen umgreifen das mediane Cytostom und ein

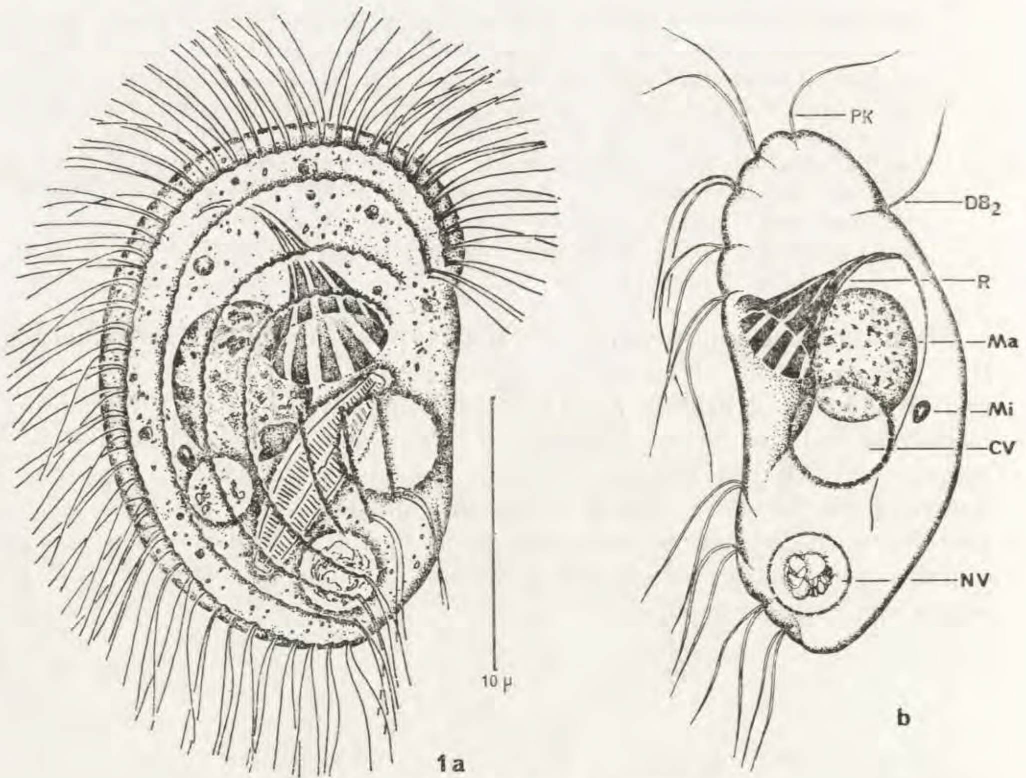

Abb. 1. *Pseudochlamydonella rheophila* sp. n.

Ventralansicht (a) und Seitenansicht (b) nach Lebendbeobachtungen CV — kontraktile Vakuole, DB — Dorsalbürste, Ma — Makronucleus, Mi — Mikronucleus, NV — Nahrungsvakuole, PK — periphere Kinete, R — Reuse

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postorales Wimperfeld. Dorsal und der Vorderkante die periphere Kinete, dahinter in einer Depression die Dorsalalbürste. Der Reusenapparat gleicht im Bau jener von *Chilodonella*. Er ist dorsalwärts nach vorne, dann abwinkelnd nach hinten gebogen.

Typusart: *Pseudochlamydonella rheophila* sp. n.

Pseudochlamydonella rheophila sp. n. (Abb. 1, 2)

In vivo 17–26 μm × 10–17 μm, Gestalt konstant, linke Seite schwach konkav, rechts ausgeprägt konvex. Dorsal gewölbt, ventral mehr oder weniger flach, je nach Ernährungszustand. Kontraktile Vakuole zentral, links unter dem Cytostom. Cilien etwa 5 μm lang. Die einzelnen Kineten in Furchen, weshalb der Ciliat von der Seite betrachtet deutlich gerippt erscheint. Cytostom in Zellmitte. Reusenapparat aus etwa 10 kräftigen Trichiten, die zunächst nach vorn, dann um den Makronucleus herum nach hinten ziehen. Einheitlicher, homogener Makronucleus, häufig in bis zu 4 kugelige Fragmente geteilt.

7 ventrale Kineten aus Dikinetiden: 4 von ihnen von links präoral nach rechts caudal ziehend und 3 kurze Kineten postoral. Dorsal, am vorderen Rand die periphere Kinete. Abhängig von der Zellteilung kann sie auch fehlen. In weitem Abstand dahinter die Dorsalbürste aus 6 bis 9 einzelnen Basalkörpern.

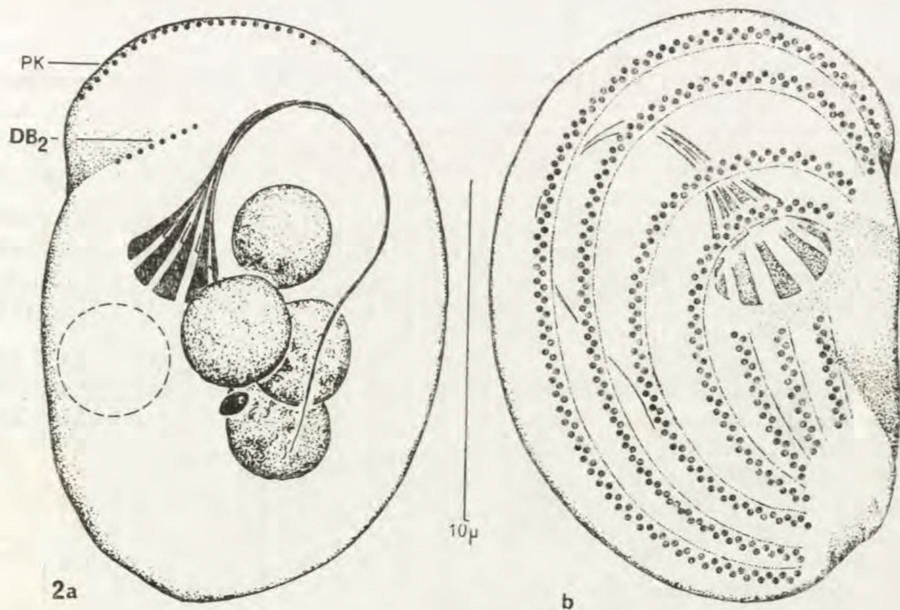

Abb. 2. *Pseudochlamydonella rheophila* sp. n.
Infraciliatur der Dorsal- (a) und Ventralseite (b) nach Protargolimprägnation DB — Dorsalbürste, PK — periphere Kinete

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Tabelle 1
Pseudochlamydonella rheophila sp. n.
Milieuspektrum:

Temperatur	°C	4,2–15,4
pH-Wert		7,6–8,2
Leitfähigkeit	mS × m^{-1}	83–420
Chlorid (Cl^-)	mg/l	82–124
Sulfat ($SO_4^=$)	mg/l	20–21
Sauerstoff (O_2)	mg/l	8,6–11,8
Kaliumpermagnat-vebrauch ($KMNO_4$)	mg/l	3,9–4,7
TOC (C)	mg/l	0,54
Phosphat (PO_4)	mg/l	≤ 0,02–0,2
Ammonium (NH_4-N)	mg/l	≤ 0,01–0,1
Nitrit (NO_2-N)	mg/l	≥ 0,01–0,01
Nitrat (NO_3-N)	mg/l	4,1–8,2
Calcium (Ca)	mg/l	86,8–88,5
Magnesium (Mg)	mg/l	11,9–12,2
Kalium (K)	mg/l	3,6
Natrium (Na)	mg/l	65–80
Eisen ges. (Fe)	mg/l	≤ 0,1
Mangan (Mn)	mg/l	≤ 0,05

Tabelle 2
Biometrische Charakteristik von *Pseudochlamydonella rheophila* Alle Daten von protargolimprägnierten Individuen

Merkmal	Min	Max	*x*	*S*	*Sx*	*V*	*n*
Länge in μm	18	25	21,2	2,19	0,53	10,3	17
Breite in μm	11	16	13,4	1,47	0,34	10,6	17
Länge des Makronucleus in μm für 1-kern Individuen	5	7	6,1	0,78	0,19	12,8	17
Breite des Makronucleus in μm für 1-kern Individuen	4	6	5,2	0,64	0,15	12,3	17
Anzahl der Basalkörper der Dorsalbürste	6	9	6,9	0,86	0,21	12,5	17
Anzahl der Makronucleusteile	1	4	1,9	1,27	0,31	67,4	17
Anzahl der Mikronuclei	1	1	1	0	0	0	9
Anzahl der postoralen Kineten	3	3	3	0	0	0	17
Anzahl der perioralen Kineten	4	5	4,2	0,39	0,10	9,4	17
Anzahl der Basalkörperpaare in 10 μm	13	17	14,6	1,37	0,33	9,4	17
Anzahl der Trichiten	8	11	8,8	0,95	0,23	10,8	17
Anzahl der Basalkörperpaare der 1.postoralen Kinete	5	7	5,5	0,78	0,19	14,0	13

n — Anzahl der untersuchten individuen, *X* — Mittelwert, *S* — Mittlerer Fehler des Mittelwertes, *Sx* — Standardabweichung, *V* — Variablitätsabweichung

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Angaben zur Autökologie

Pseudochlamydonella rheophila kommt regelmäßig in den Quellen der Alme, einem Fließgewässer im Sauerland, vor. Der größte Teil des Quellgebietes — mit 104 Einzelquellen das bedeutedsste des Briloner Kalkmassives- wird in limnologischer Hinsicht vom Typ der Limnokrene repräsentiert. Der Gesamtabfluß aus allen Quellen beträgt etwa 400 l/s. Der Chemismus des Quellwassers wird entscheidend durch die geologischen Verhältnisse geprägt. Örtliche Unterschiede im Temperatur- und Chloridgehalt sind in dem ca. 1 ha großen Gebiet feststellbar, doch jahreszeitlich bedingte Schwankungen der die Wasserqualität kennzeichnenden Parameter treten kaum auf. Das Alme-Quellwasser in seiner Gesamtheit kann als Calziumbikarbonat- Natriumchlorid- Magnesiumsulfat- Wasser charakterisiert werden (Analyse des Quellwassers vom Staatlichen Amt für Wasser und Abfallwirtschaft, Lippstadt. Tab. 1, 2).

Pseudochlamydonella rheophila ist ein typischer Aufwuchsbewohner, der bei Beunruhigung haptisch reagiert, indem er sich fest ans Substrat heftet. Der Aufwuchs, in dem der Ciliat vorkommt, ist in der Hauptsache von Kieselalgen dominiert. Hohe Bestandsdichten erreichen *Achnanthes lanceolata, Achnanthes minutissima, Cocconeis placentula, Diatoma hiemale* und *Meridion circulare*. Die kleinsten Formen wie *A. lanceolata* und A. *minutissima* sind die Hauptnahrung, Das Abundanzmaximum der Diatomeen im Frühjahr ist daher mit einem Populationsmaximum des Ciliaten korreliert. Andere vagile Ciliaten, meist in größerer Abundanz, treten als Nahrungskonkurrenten auf: *Gastronauta clatratus* Deroux *Chilodonella uncinata* Ehrenberg, *Chlamydonella pseudochilodon* Deroux und *Glaucoma* spec. An räuberischen Begleitformen wurden hauptsächlich gefunden: *Litonotus cygnus* O.F.M., *Loxophyllum meleagris* Dujardin., *Lacrymaria olor* O.F.M. und eine *Sphaerophrya* spec. *Pseudochlamydonella rheophila* ist kaltstenotherm, ähnlich wie die Begleitarten *Gastronauta clatratus* Deroux und *Chlamydonella pseudochilodon* Deroux. *Pseudochilodonella rheophila* kommt auch in den Waldbächen des Bonner Kottenforstes vor. Nach von Jutrczenki (1978) sind dies oligo- bis β- mesosaprobe Gewässer mit einer jährlichen Temperaturamplitude von etwa 4–15°C.

Diskussion

Ein kennzeichnendes Merkmal vieler *Cyrtophorida* ist die von Deroux (1970, 1976 a,b) so bezeichnete "cinétie droite externe" (cde), ein System von Kineten, das aus einem ventralen und einem bis drei dorsalen

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Anteilen bestehen kann. Die dorsalen Teile ist die Dorsalbürste dieser Ciliaten. Bei der Teilung entstehen die Dorsalbürsten aus Kinetosomen, die vom ventralen cde-Teil proliferieren. Dies hat zur Folge, daß der Aspekt der cde abhängig vom Teilungszustand ausgesprochen variabel sein kann. Dies trifft hier auf die periphere Kinete zu. Die neue Familie *Pseudochlamydonellidae* hat ein ventrales Kinetom ans gepaarten, zickzackförmig stehenden Kinetosomen. Die Infraciliatur der Kineten, das Fehlen circumoraler und praeoraler Kineten sind die familientypischen Merkmale, welche die *Pseudochlamydodontidae* von den anderen Familien der Unterordnung *Chlamydodontina* (*Chilodonellidae, Chlamydodontidae, Lynchellidae*) unterscheidet.

SUMMARY

Pseudochlamydonella rheophila sp. n., gen. n., fam. n., a new ciliate from the periphyton in brooks in Nordhein-Westfalen (FRG) is described. The typical pattern of the infraciliature: 7 ventral kineties — 4 ones on the right going in a semicircle around the cytostome, 3 postoral ones. The "cde" is apically located. The oral aperture is rounded and supported by strongly developed trichites. There is one dorsal brush. Character of the new family: the kinetosomes in the kineties are arranged in a zigzag, preoral and circumoral kineties are absent.

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Ciliates Associations on the Body of Krill (*Euphausia superba* Dana)

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Synopsis. A population of *Euphausia superba* Dana which occurred in the Antarctic south of Australia was infested by Ciliates; in average 55% by *Ephelotidae* and in 100% by *Apostomatida*. Several to several dozen *Ephelotidae* and few hundred *Apostomatida* were found on individual specimens of *E. superba*. *Ephelotidae* occurred most numerously on the ventral side of the abdomen between the 2-nd and 4-th pleopods, while *Apostomatida* were found on the body on the thoracic limbs and on the pleopods and on setae. Within both protozoans which belong to different taxonomic groups of *Ciliophora* a number of forms were found which varried in size and body structure: they infested krill in different degrees and in different places.

Only some papers and one note pertain to protozoans epibionts associated with the *Euphausiidae*. Nicol (1984) noted the presence of *Ephelota* sp. on the body of *Meganyctiphanes norvegica* in the Bay of Fundy. Stawiszyńska-Janas and Kittel (1982) reported *Ephelota* sp. infesting krill in the Admiralty Bay (King George, South Shetland Islands) and Rakusa-Suszczewski and Filcek (1988) reported *Apostomatida* infesting krill during the year. Lindley (1978) found that nine north-Atlantic species of *Euphausiidae* were infested by *Apostomatida*. Both these ciliates are also known as epibionts on copepods, amphipods and decapods (Sewell 1953, Sherman and Schaner 1965, Sawyer et al. 1976). The present work is to call attention to the common presence of protozoans on the bodies of Antarctic krill.

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Methods

Krill were caught during the Japanese SIBEX-BIOMASS 1983–84 expedition of the vessel Hakuhò-Maru south of Australia (Fig. 1). We have examined samples of *E. superba* obtained at three stations: G-2 (65°32'S. 149°57.1'E) 22 December 1983, IKMT net; 5 (64°55.5'S, 117°59.1'E) 20 January 1984, IKMT net; P-I-3-4 (64°38.4'S, 127°09.8'E) 18 January 1984, ORI-net.

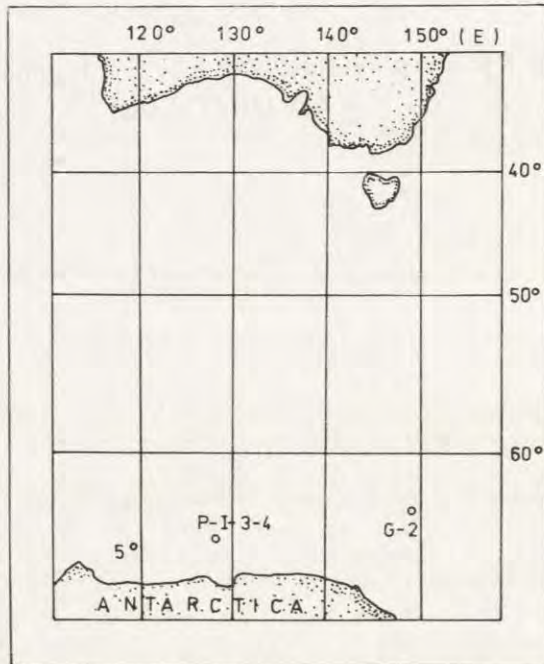

Fig. 1. Chosen Japanese SIBEX-BIOMASS stations (1983/84) where krill infested by protozoans were caught

The length of *E. superba* was measured from rostrum to the end of telson with the accurracy of 1 mm. The surface of the body was examined under binocular and the number of protozoa of the family *Ephelotidae* was counted. Altogether 100 specimens from station G-2, 142 from station 5-I-3-3-4 and 148 from station 5 were measured and examined. Since the material we worked with had been earlier used, the number of *Ephelotidae* which are presented here might be underestimated, because of the easy way that protozoa break off from the body of the host. In the present work which has been done on the material preserved by formalin we have decided not to distinguish the genera and species of the protozoa. Description of the new species is possible only after the recognition of its life-cycles.

In the second part of the investigation ten specimens of *E. superba* were taken at random from each of the station: 5, P-I-3-4, and they were examined under microscope. Among the 3 forms of protozoans, which belong to *Apostomatida* (cf. Rakusa-Suszczewski and Filcek 1988), the most numerous form No. 1 was counted.

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In order to calculate the volume of forms 1, 2, 3 a plastic clay model was made identical with the shape and size of protozoans; it was reshaped into a ball to measure its diameter and volume (in μm^3). The systematic terminology of the investigated protozoa has been adapted de Puytorac et al. (1984) and Kudo (1977).

Results

The boby of *Euphausia superba* Dana were infested by protozoans belonging to two groups: genus *Ephelota* (*Ephelotidae*, *Suctorida*) and family *Foettingeriidae (Apostomatida)*. *E. superba* is a host to at last three species with characteristics of the genera *Ephelota*.

The smallest *Ephelota* had the following measurements: body width ca. 100 µm; width of stalk at the connection with body ca. 75 µm; length of prehensile tentacles 100-125 µm; length of suctorial tentacles at the apex of the body about 25 µm.

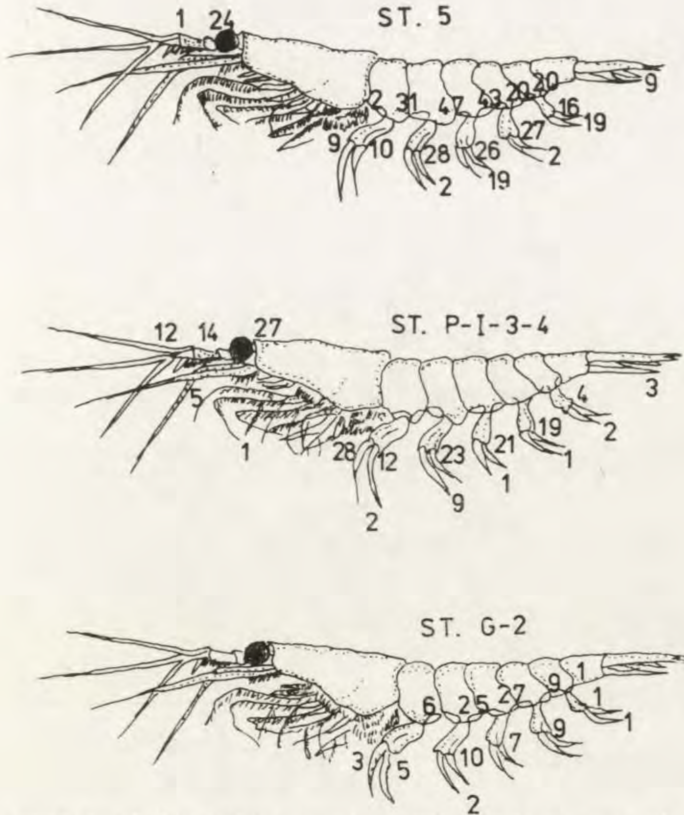

Fig. 2. Total numbers of *Ephelotidae* found on different body parts of all the examined individuals of *E. superba*

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Medium size *Ephelota* had: body width 200–250 μm; body height 150–175 μm; length of prehensile tentacles ca. 175 μm; length of stalk 500-750 μm. Disc at the point of attachment to *E. superba* had a diameter 35 μm and stalk width was 25 μm.

The lagest *Ephelota* had: body width 450–500 μm, body height 250–350 μm, a distinct depression at the apex, length of stalk to 1 mm, flattaned body. Some individuals of *Ephelota* were in a state of reproduction through the characteristic exogenous budding. Larvae of the largest *Ephelota* forms had a diameter reaching 90 μm and ciliae ca. 15 μm.

On the body of *E. superba* from station G-2 a single specimen of protozoa was observed having ad different body structure from that of the genera *Ephelota*. It was half-moon shaped transversely flattened, the width was 225 μm, height in the central part 100 μm. Prehensile tentacles were short and thick (1 μm) at the base. Suctorial tentacles were thick and pipe-like. Stalk consisted of a double ring; the part attached

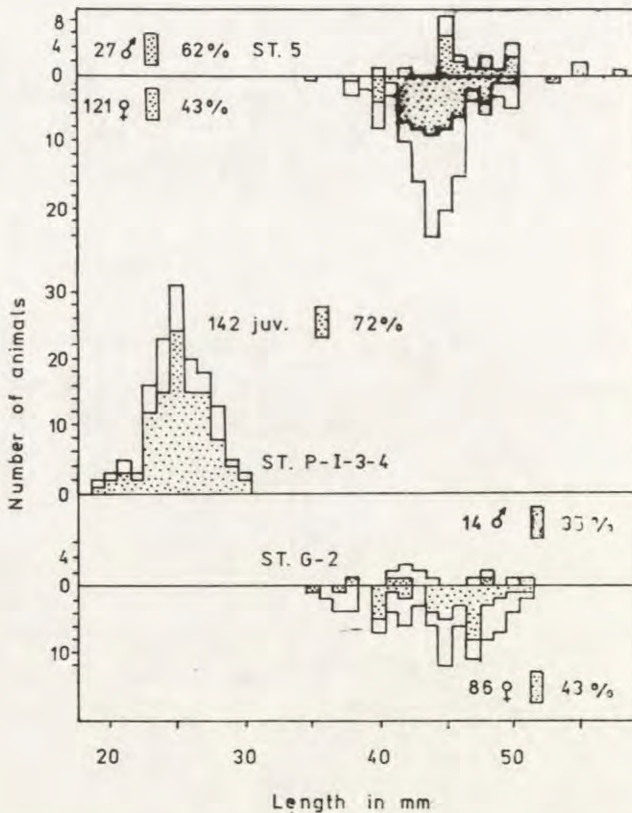

Fig. 3. Length distribution of the population of *E. superba* and infestation by *Ephelota* (dotted bars) in different length classes of krill

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to the host had vertical stripes, was darked, and had a diameter 25 μm. Disc with a diameter 60 μm, was surrounded by thick ring.

Distribution of *Ephelota* on the body surface of *E. superba* is very characteristic (of. Fig. 2). Mostly, the individuals are attached to the ventral side of the body and the inner part of the tergites of abdomen between the 2-nd and 4-th pleopods, as well as on the basipodite and endopodites 2, 3, 4 of pleopods. They are rarely found on the exopodites.

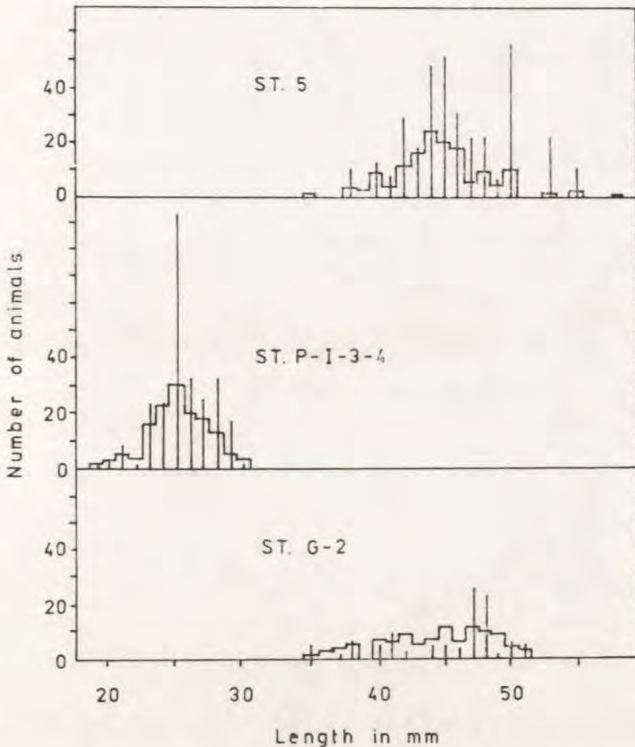

Fig. 4. Length distribution of *E. superba* and intensity of infestation (lines-number of *Ephelota*) in relation to length classes of krill population

A similar distribution of *Ephelotidae* on *E.superba,* both on juvenile and adult forms of krill was ascertained at three investigated station (Fig. 3). Altogether, of the 384 individuals of *E superba* with size between 19 and 58 mm more than 55% (35% to 72%) were infested with *Ephelotidae.* A greater infestation was found in a jounger population of *E. superba,* where the numbers of *Ephelotidae* would approach sometimes several dozen on a single specimen of krill. On the average, one specimen of krill was host to a few *Ephelotidae* (Fig. 4).

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Protozoans of the family *Foettingeriidae* were found on the body surface of *E. superba*. Hundred percentage of krill examined from three stations were infested by forms 1, 2, 3 (described by Rakusa-Suszczewski and Filcek 1988) belonging most likely of the phoron stage (resting stage). On the body of krill the most frequently occurring

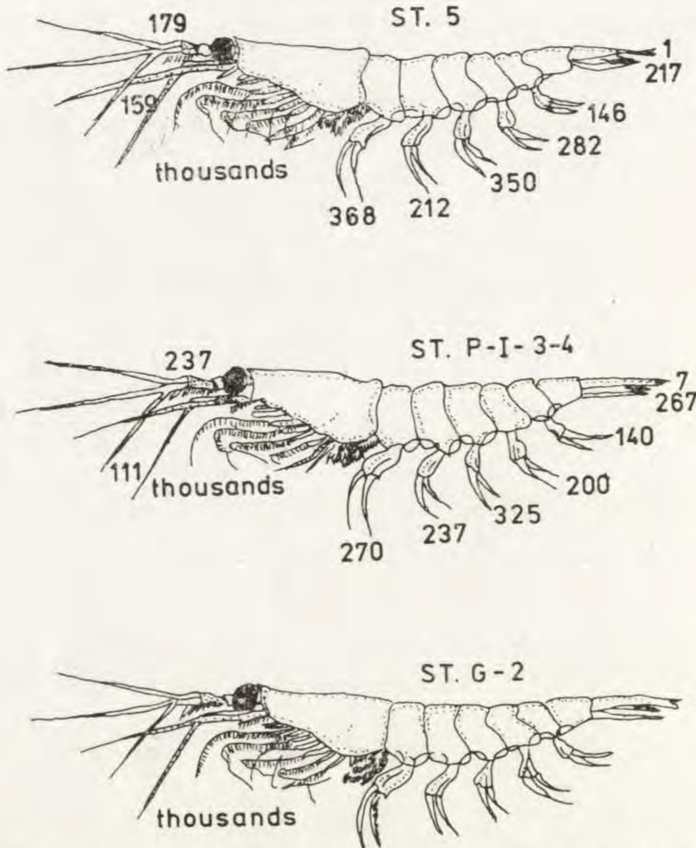

Fig. 5. Total numbers of *Apostomatida* found on the different body parts of *E. superba* in all individuals examined

were forms 1. It occurs in groups mainly on the distal segments of endopodites; found less frequently on exopodites of thoracic limbs, endopodites and exopodites of pleopods and uropods, on the antennules, antennae and uropods (Fig. 5). Form 1 has the length 35–45 μm and body volume is about 9800 μm^3. Form 2 has the length 75–87 μm and body volume about 30 000 μm^3 occurred more frequent on the plumose setae exopodites than on endopodites of thoracic limbs. Form 3 has a round base and body height 35–50 μm, volume about 11 000 μm^3 occurred singly in place devid of setae. Only form 1 was counted on the body of krill.

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Discussion

The few works about Antarctic *Protozoa* which have been published pertain mainly to parasites of fish (Noble 1984) or amphipods (Lipa and Rakusa-Suszczewski 1980). In spite of the many investigations on krill conducted so far little attention has been paid to the infestation of *E. superba* by protozoan epibionts (cf. Rakusa-Suszczewski and Filcek 1988). As shown by the present results, more then 55% of individuals of *E. superba* at three off shore stations were inhabited by *Ephelotidae* and 100% by *Apostomatida*. The observed infestation of the krill also strongly depends on moulting but because our materials were preserved, we can not to make any farther conclusions.

Ephelotidae occur most numerously between 2-nd and 4-th pleopods settlement on the ventral side of krill body, in places washed by the strongest water current during movement of krill (cf. Fig. 6). Undoubtedly there are currents close to the body surface, between the pleopods which enable the attachment of the free swimming larvae of *Ephelotidae*.

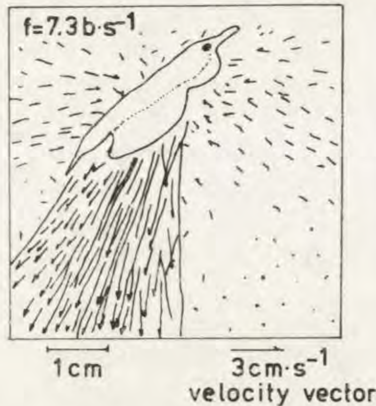

Fig. 6. Redrawn from Kils (1981) Fig. 11, flow field of an intense pleopods best during swimming of *E. superba*. The length of the arrows is a measure for the flowing speed. The thick line indicate the flow boundary of the "propulsion stream"

Foettingeriidae occur on appendages which are filtering water very intensively (thoracic limbs and pleopods on setae and between them). They are also found at joints of segments, antennules and antennae, in the area of rostrum and in depressions of carapace where microcurrents are formed which facilitate the attachment of protozoans.

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Ephelotidae and *Foettingeriidae* belong to different groups within *Ciliophora* (de Puytorac et al. 1984). Both grups of protozoans infested on the different parts of the body of krill.

As reported by Stawiszyńska-Janas and Kittel (1982) on average of 18% of *E. superba* and 52% *E.crystallorophias* from the Admiralty Bay were infested by *Ephelota* sp. *E. superba* from Admiralty Bay was infested also by *Apostomatidae* (Rakusa-Suszczewski and Filcek 1988). These results show that infestation of *E. superba* by both group of protozoans is a circum-Antarctic phenomenon and it occurs equally in the nearshore zone of the South Shetland Islands and in an offshore area south of Australia.

Ephelotidae are reproducing by free slowly-swimming larvae (Kudo 1977) which produce a stalk after reaching the host. The high infestation found in *E. superba* is undoubtedly related to the formation of swarms by this species.

In the northern hemisphere Nicol (1984) found that 8 to 82% of *M. norvegica* in the Bay of Fundy was infested by *Ephelota* sp.

Infestation of *E. superba* by *Apostomatida* was much greater in the Antarctic than that observed by Lindley (1978) for the north-Atlantic *Euphausiidae* (3.4% to 16% of individuals infested). Lindley (1978) also observed seasonal differences in infestation and its relationship to the length of host and a higher infestation in the inshore zone. In Antarctic the numbers of forms 1 and 2 found on the body of *E. superba* are greater in autumn than in spring (Rakusa-Suszczewski and Filcek 1988). Protozoans as a epibionts of the krill is undoubtedly an important, but not investigated so far element of the Antarctic ecosystem.

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Bacteria in Krill (*Euphausia superba* Dana) Stomach

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Synopsis. Number of bacteria counted in *Euphausia superba* Dana stomach is about 260 thousand times higher than that in surrounding sea water. It may suggest that the interior of stomach creates favourable conditions for bacteria many of which are at the stage of dividing. The surface of krill body is relatively free of bacteria.

The total count of free-living bacteria in water of the surroundings of krill in the Antarctic is of an order of 10^6–10^8 per dm^3 (Hodson et al. 1981, Kogura et al. 1985, Zdanowski 1985, Bailiff et al. 1987). The major part of these bacteria belong among osmotrophic planktobacteria with low affinity to nutrient-rich surfaces (Sieburth 1979). The minor part of bacteria (of an order of 10^2–10^4 CFU per dm^3, Zdanowski 1985), representing in free-living form "starving transients" (Sieburth 1979), are classed among osmotrophic epibacteria with great affinity to surfaces. Probably only the bigger ($\geqslant 1$ μm) free-living bacteria, being scarce in the Antarctic sea ecosystem, can be taken up by krill directly from outside by filtration (cf. Marschall 1985). The most frequent, fine (0.2–0.6 μm) osmotrophic planctobacteria, unavailable by this route, can be taken up provided that they have earlier been ingested by protozooplankton. Osmotrophic epibacteria occurring in the form of aggregates on the surface of living sea organisms (Sieburth 1979, Rakusa-Suszczewski 1988) and in detritus (Zdanowski 1981) have a much greater chance of landing directly in krill stomach. The bacteria in the krill stomach can be a source of enzymes cooperating with the highly specific enzymes of krill (Turkiewicz et al. 1985, Mayzaud et al. 1987) in digestion of very differentiated natural and experimental food (diatoms, protozoa, dinoflagellata, chlorella,

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zooplankton, other *Euphausia* or *Artemia* eggs) and in the autoproteolytic processes (Kołakowski 1986). It seems that the part played by bacteria in krill nutrition is more important than it has so far been assumed.

Materials and Methods

The 21 *Euphausia superba* Dana individuals caught in the vicinity of Elephant Island in January 1984 were analyzed. The total bacterial count (TC) was determined of 21 krill stomachs. The mean wet weight of krill was 430 mg ± 132 mg and the mean wet weight of a stomach was 2.47 ± 0.78 mg. The stomachs examined were evidently filled with food. Stomachs were homogenized in 10 ml of redistilled water in a glass homogenizer with a Teflon shaft (Zdanowski 1981). The homogenate was centrifuged at 200 g for 1 min to separate the bigger remnants. The supernatant was filtered through polyearbonate filters (pore size 0.2 μm). TC was determined under an epifluorescence microscope (EFM) after staining with acridine orange (Zimmerman and Meyer-Reil 1974, Hobbie et al. 1977). Also, SEM micrographs were taken of bacteria from stomach contents of krill caught in January 1987 in the vicinity of Elephant Is. Bacteria settled on millipore filters (pore size 0.2 μm) were not derived from homogenate, but originated from stomach contents taken with a microcapillary tube.

Results and Discussion

The mean krill stomach weighing 2.47 ± 0.78 mg was found to contain 1.4×10^{-7} of bacteria which is on the average 260 thousand times more than the surrounding sea water, when calculated per volume unit. The major part of stomach-derived bacteria were spherical and were about 1 μm in diameter. If it is assumed: that krill filters only particles greater than 1 μm (Boyd et al. 1984, Marschall 1985), that the major part of free-living bacteria in sea water are much smaller in diameter, and that the volume of water filtered is only 0.36 dm^3 per hour and per individual (Quetin and Ross 1985), then the efficiency of filtering seems to be too low to account for the high concentration — in the stomach — of bacteria derived from the water column. So high a concentration of bacteria (less than 1 μm in diameter) must be largely due to *in situ* growth of bacteria developing on the nutrient rich stomach content of krill (Pl. I. 1 a, b). The biomass of bacteria seems to be small, and thus of low significance as a source of food. On the other hand, the total surface of bacterial cells is enormous. Such a population may play an important part as e.g., a source of amino acids or vitamins or as a so-

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urce of enzymes co-operating with krill enzymes in digestion of the greatly differentiated plant and animal food of krill. Particularly in winter the importance of the bacteria in krill nutrition may grow. The bacteria were found also on krill carapace but not very abundante. Individual bacteria or small colony attached the surface of krill.

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EXPLANATION OF PLATE I

1 a, b: Scanning electron micrographs of bacteria from krill stomach content. The material was collected from krill stomach with a micropipette and prepared for SEM on an Acrodisc Filter (pore size 0.2 μm)

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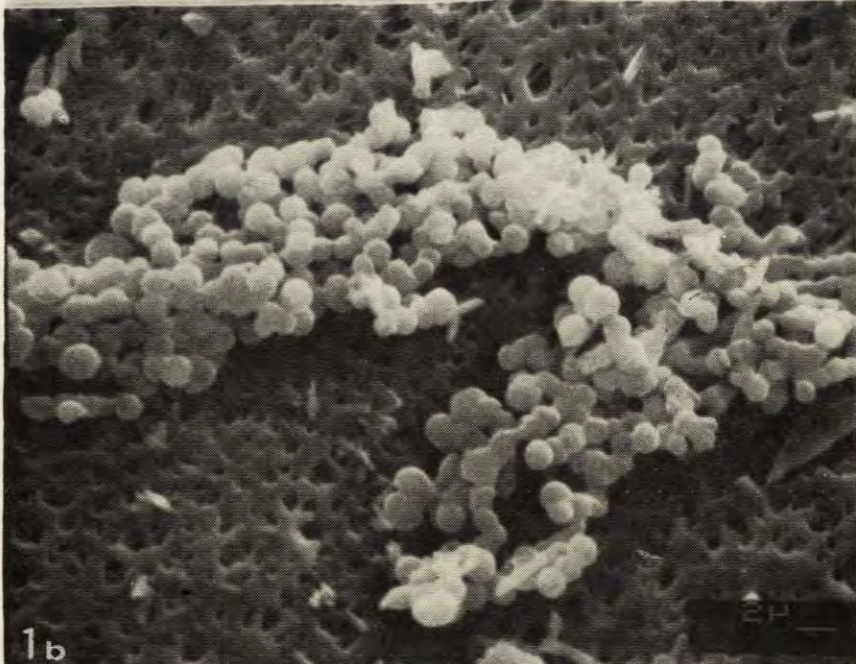

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M. Jerka-Dziadosz, K. Muszyńska and M. Frontczak: Genetic Analysis of Ciliary Pattern Mutants in *Paraurostyla weissei* — P. Alonso and E. Zubiaur: Determination of Free and Bound Amino Acids in Three Strains of *Naegleria* — I. R. Isquith, J. Swenson, W. C. Kleinelp and J. George: Locomotor Response of Five Species of Ciliates to Magnetic and Electrical Stimuli in Varied Chemical Environments — E. Wyroba: Pharmacological Specificity of Beta Blockers-induced Inhibition of Paramecium Phagocytosis — L. Szablewski: Recovery of *Tetrahymena pyriformis* from the Effects of Colistin. I. A Fluorescence Study — L. Szablewski: Recovery of *Tetrahymena pyriformis* from the Effects of Coliston. II. A Phagocytosis Study — L. Szablewski: The Effect of Pretreatment with Colistin and Cycloheximide on the Albumin Uptake in *Tetrahymena pyriformis* — P. S. Dhanaraj, B. R. Kaushal and Rup Lal: Effects on Uptake by and Metabolism of Aldrin and Phorate in a Protozoan, *Tetrahymena pyriformis* — S. I. Beylina, M. Cieślawska, B. Hrebenda and Z. Baranowski: The Relationship Between the Respiratory Rate and the Period of the Contraction-Relaxation Cycle in Plasmodia of *Physarum polycephalum* — G. Csaba, P. Kovács and V. László: Impact of the Simultaneous and Successive Imprinting of Different Peptide Molecules on Receptor "Memory" — G. Csaba and L. Kohidai: Interrelationships of Hormone Concentration, Hormonal Imprinting and Receptor Down-regulation in *Tetrahymena* — G. Ghose: First Record on Microsporan Infection on *Sitophilus oryzae* (L.) in India — D. Dutta and G. R. Sapra: A Simple Method for Production of Amicronucleate Cell Lines in Ciliates by cis.-Dichlorodiammineplatinum II

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