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Review Article

# Protozoa as Model System for Studies of Sensory Light Transduction: Photophobic Response in the Ciliate *Stentor* and *Blepharisma*

**Hanna FABCZAK**

**Department of Cell Biology, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Warszawa, Poland**

**Summary.** *Stentor coeruleus* and the related *Blepharisma japonicum* possess photoreceptor systems that render the cells capable of avoiding light. On account of this unique feature, these ciliates exhibit photodispersal as they tend to swim away from a bright illumination and accumulate in shady or dark areas. The observed photobehaviour is largely the result of a step-up photophobic response displayed by both ciliates, although other behavioral reactions like phototaxis or photokinesis may also contribute to the photodispersal. The photophobic response caused by a sudden increase in light intensity (light stimulus) starts with a delayed cessation of ciliary beating that results in the disappearance of the cells forward swimming, then a period of ciliary reversal (backward movement) followed finally by renewed forward movement, often in a new direction. Reversal of ciliary beating during the photophobic response correlates with the generation of an action potential. The action potential is elicited by a photoreceptor potential, a transient membrane depolarization produced by the light stimulus. The photoreceptor potentials in both ciliates are initiated by light absorption in a cellular photoreceptor system based on hypericin-like chromophores - blepharismin in *Blepharisma* and stentorin in *Stentor*. Recent evidence indicates that biochemical processes, which couple the photochemical cycle within the cell pigment with photoreceptor potential, may be different in these organisms. In the case of *Stentor,* cyclic GMP is the probable candidate for an internal second messenger in photosignal transduction. In related *Blepharisma* cells, however, $InsP_3$ seems to be responsible for the alterations in membrane potentials and induction of light avoiding response. The data show that lower eukaryotic cells may use similar signal transduction pathways as observed in multicellular organisms. Therefore, on the basis of light-dependent events observed in *Blepharisma* and *Stentor,* it seems appropriate to use protozoan cells as a model system for multidisciplinary studies of sensory signal transduction within single cells.

**Key words:** *Blepharisma japonicum*, cGMP, cGMP-dependent ion channels, ciliates, membrane potentials, G-protein, $InsP_3$, photophobic response, photoreceptor system, phototransduction, *Stentor coeruleus*.

## INTRODUCTION

The avoidance of brightly illuminated regions and gathering in shady places by two closely related ciliates, blue-green *Stentor coeruleus* and pink *Blepharisma japonicum,* was described for the first time at the start of this century (Jennings 1906, Mast 1906). This phenomenon, called photodispersal, is a possibility by the cells to perceive changes in the level of light intensity in their environment and to react to these differences by changing their pattern of movement (Diehn *et al.* 1977).

Among these behavioural reactions, there is only a phototactic response described so far for *Stentor*, which is observed when the ciliates are given a light stimulus

Address for correspondence: Hanna Fabczak, Department of Cell Biology, Nencki Institute of Experimental Biology, 02-093 Warszawa, ul. Pasteura 3, Poland; E-mail: hannafab@nencki.gov.pl

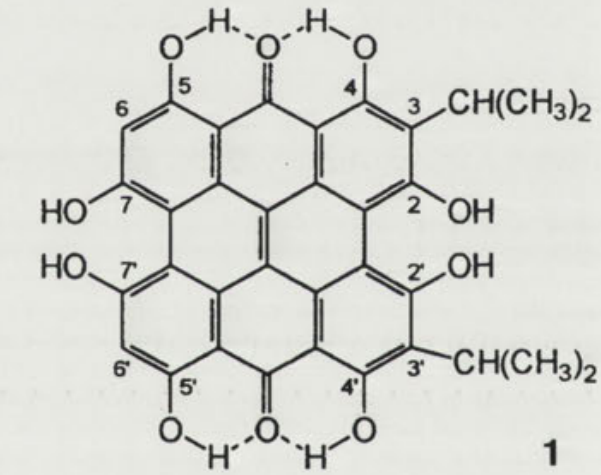
from a particular direction. Under these light conditions, the cells turn and swim away from the light source, along the direction of light propagation (Song *et al.* 1980b). The other form of photobehavioral reaction, which occurs in both ciliates and leads to photodispersal, is an increased speed of cell movement in a more intensively lighted environment (Kraml and Marwan 1983; Matsuoka 1983a,b; Iwatsuki 1991). The best described motile reaction, which is most important in causing the photodispersal effect, is the step-up photophobic response. This photoreaction occurs in a similar way in *Blepharisma* or *Stentor* and consists of a change of direction of cell movement as a result of the temporary reversal of ciliary beating when the cell swims from the shaded area to a brightly illuminated region (Wood 1976, Song *et al.* 1980a, Kraml and Marwan 1983, Fabczak and Fabczak 1995).

The motile photobehavior of *Blepharisma* and *Stentor*, unique among ciliates, are caused, as it is thought, by the presence of numerous specific high-coloured granules in the cell cortex. These granules contain a pigment, which is supposed to play the role of a photoreceptor and is called blepharismin in *Blepharisma* and stentorin in *Stentor*. The physiological reasons why both ciliates avoid lighted areas are not clear at present. It is known that prolonged cell exposure to intensive light levels in the visible range causes significant disturbances in the occurrence of the photophobic response in both ciliates and can even lead to their death as a result of the photodynamic effect. It is blepharismin and stentorin that are the cause of this phenomenon because, being derivatives of hypericin, they act as a strong photosensitizer. Hypericin, synthesized by some plants, is known as a natural photosensitizer causing hypericism in animals, a state of high skin sensitivity to light in animals as a result of the ingestion of hypericin-containing plants and feed. In the case of ciliates, the photophobic response can be a safety measure for cells protecting them from photodynamic effect (Falk 1999). In *Blepharisma*, it was also observed that during an attack of a predator, for example another ciliate *Dileptus*, there is a quick discharge of the contents of the pigment granules, which causes an immediate repelling of the aggressor because blepharismin has toxic properties (Harumoto *et al.* 1998). The photodispersal observed in both ciliates assures the main presence of these organisms in poorly lighted areas that enables maintenance of a high level of blepharismin in cells and thus creates optimal conditions for ciliate survival (Harumoto *et al.* 1998).

**light ⇒ ????? ⇒ photodispersal**

## PHOTORECEPTOR SYSTEM

Studies of cell photopigments, stentorin in *Stentor* ciliates, started at the end of 19th century (Lankester 1873) and on blepharismin, in the related ciliate *Blepharisma* (originally called zoopurin), were initiated significantly later (Arcichovsky 1905). Both these pigments are located exclusively in pigment granules (Randall and Jackson 1958, Meza-Keuthen 1992, Matsuoka



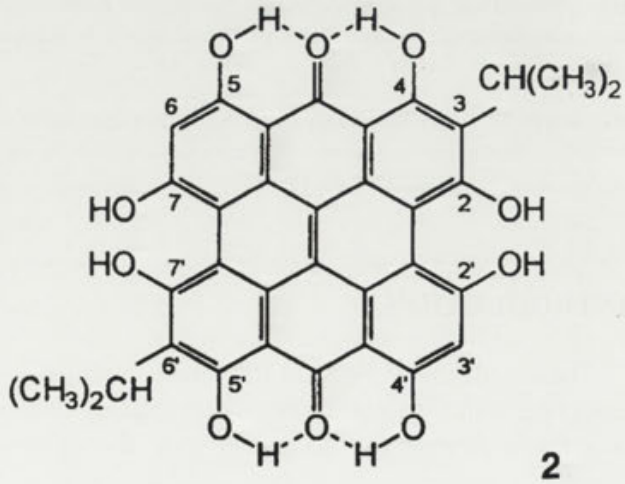




**Fig. 1.** Possible structure of stentorin (from Tao *et al.* 1994)

*et al.*1993, Tao *et al.* 1994) and are densely packed between kinets of cell cortex layers (Inaba *et al.*1958, Kennedy 1965, Giese 1973, Huang and Pitelka 1973, Newman 1974, Matsuoka *et al.* 1993). The stentorin as well as blepharismin have been proposed to be hypericin-like molecules based on the similarities in spectroscopic characteristics. Stentorin and blepharismin belong to the mesonaphtodianthrone class of compounds (Fig. 1) and constitute a new class of cell photoreceptors which are significantly distinct from other, already well known photoreceptors, such as rhodopsin, bacteriorhodopsin, phytochrom, chlorophyll and flavines (Møller 1962, Sevenants 1965, Song *et al* 1990, Checcucci *et al.*1993, Tao *et al.* 1994, Matsuoka *et al.* 1997, Song 1997a, Falk 1999).

Though photochemical processes occurring within the cell pigment elicited by light absorption are not fully known, it is thought that, in analogy to much better known photoreceptors of higher organisms, electrons and/or proton transfer may be involved in the primary photoresponses of ciliate photoreceptors (Song 1997a, Wells *et al.* 1997, Angelini *et al.* 1998, Falk 1999, Losi *et al.* 1999).

Two groups of pigments, stentorin 1 and stentorin 2, exist in *Stentor* (Kim *et al.* 1990). Stentorin 1 is strongly fluorescent and appeared to be a relatively small complex composed of at least two heterodimeric proteins corresponding to apparent molecular masses of 46 kD and 52 kD on 13 % SDS-PAGE. Stentorin 2 is weakly or nonfluorescent. It is a large protein complex which is eluted from a Bio-Gel A- 1.5 m column near the void volume (Song *et. al.* 1990, Tao *et al.* 1994). Recently, stentorin 2B was isolated from stentorin 2 by hydrophobic interaction chromatography. It contains a chromophore covalently bound to an approximately 50 kD apoprotein, determined by SDS-PAGE urea (Song 1995, 1997a). Blepharismin, on the other hand, is bound to a protein whose molecular mass was established as 38-50 kD on the basis of resolving SDS-PAGE (Gioffré *et al.* 1993, Yamazaki *et al.* 1993). These results were not, however, confirmed in later studies because the photoreceptor in *Blepharisma* was identified as a protein complex of significantly greater molecular mass (200 kD) (Matsuoka *et al.* 1993).

The newest investigations utilising methods of molecular biology supplied new data about the possible structure of cellular photoreceptors that can participate in the process of light absorption in ciliates. The results of these experiments show that, in *Blepharisma* and *Fabrea salina*, another ciliate in which only the photoreceptor containing hypericin-like chromophore was so far found (Marangoni *et al.* 1997, Kuhlmann 1998), there are genes present coding the opsin, apoprotein of rhodopsin, the photoreceptor typical for the cells of higher organisms. The presence of a protein homologous to rhodopsin was also found in the ciliate *Paramecium bursaria*, which is sensitive to light (Nakaoka *et al.* 1991). At the present stage of knowledge, it is difficult to judge unequivocally what kind of photoreceptor is engaged in the system of light signal processing in *Blepharisma* and *Stentor.*

**light ⇒ photopigment ⇒ ???? ⇒ photodispersal**

## STEP-UP PHOTOPHOBIC RESPONSE

In both *Stentor* and *Blepharisma* ciliates, the photophobic response to light stimulus (step-like increase in light intensity) occurs in a similar way. A few characteristic stages can be distinguished in this motile reaction: the cessation of swimming, appearing with some delay in comparison to the stimulus onset, followed by a period of cell backward movement as a result of ciliary beating reversal, after which the cell stops again, and finally starts forward swimming in a randomly chosen direction (Fig. 2).

In both ciliates, the time delay of movement response decreases and the duration of ciliary reversal increases with increasing stimulus intensity. *Stentor* reacts much faster to light stimulation; the delay equals 0.1 to 0.3 s under standard conditions, depending on the light intensity (Fabczak *et al.* 1993c), whereas in *Blepharisma* it can reach up to 1 s (Fabczak *et al.* 1993d). Attention has to be paid to the fact that both ciliates react to light with a delay much longer than the ciliary response when the same ciliates are exposed to mechanical stimulation (Wood 1982, S. Fabczak-unpublished). The significant delay in time with which the photophobic reaction occurs in *Blepharisma* and *Stentor* suggests that this time is necessary for some biochemical processes, triggered by light stimulation, to occur which lead finally to the motile photoresponse (Song 1997b).

**light ⇒ pigment ⇒ ??? ⇒ photophobic response**
**⇓**
**photodispersal**

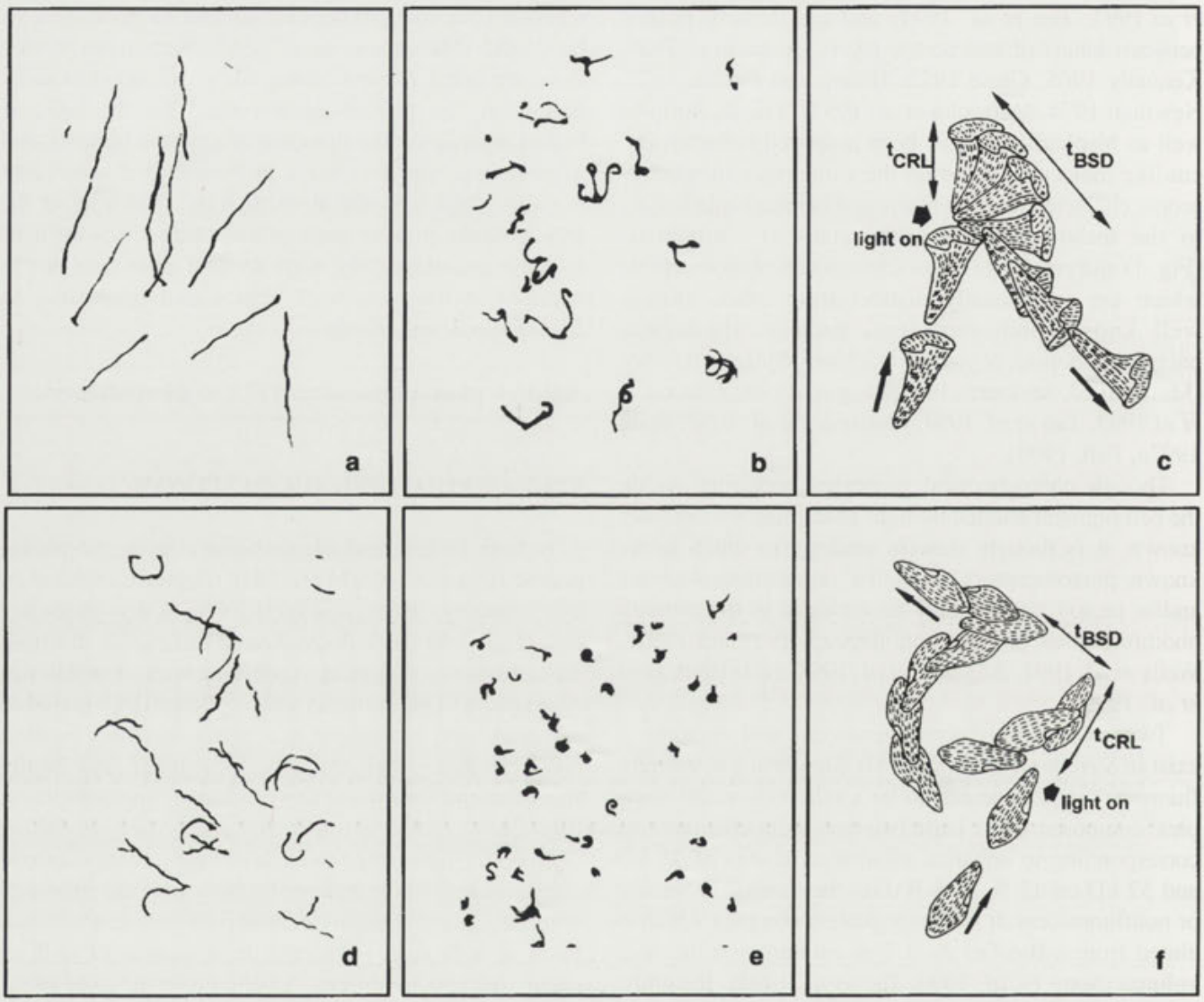



**Fig. 2.** Photophobic reaction in (**a-c**) *Stentor* and (**d-f**) *Blepharisma* ciliates: (**a**, **d**) macrophotographs of the cells motile behaviour under low light intensity, (**b**, **e**) macrophotographs of the photophobic response during light intensity increase, (**c**, **f**) schematic drawing of the photophobic response (from Fabczak and Fabczak 1995)

## MEMBRANE POTENTIAL CHANGES

### Photoreceptor and action potentials

Preliminary attempts to investigate the electric changes occurring in the protozoan cell membrane in response to light stimulation were started in *Stentor* cells, using intracellular glass microelectrodes introduced into the large vacuole (Wood 1976, 1991). These intravacuolar recordings showed that light stimulation is followed by a depolarising membrane potential. Electrophysiological experiments performed in the ciliate *Blepharisma* have also shown that light may cause similar changes in membrane potential. However these recordings were not very persuasive because of the existence of numerous different artefacts during the recordings (Colombetti *et al.* 1987).

More detailed data regarding the membrane potential changes in *Stentor* and *Blepharisma* upon light stimulation were gathered using intracellular microelectrodes, introduced to the cytoplasm of cells adapted to a lowered temperature. This procedure entirely overcame the prob-

lems connected with the recording of membrane potential in ciliates usually occurring at room temperature (Wood 1982, Fabczak and Fabczak 1988, Fabczak 1990). The recordings showed that the resting membrane potentials in *Stentor* and related *Blepharisma* adapted to darkness equals approximately - 50mV, and is generated mainly by potassium ions. The dark-adapted cells when subjected to stimulation by light of low intensity generates a gradual, depolarising photoreceptor potential, the amplitude of which increases with an increase in stimulus intensity up to maximal values in a range between 15 and 25 mV (Fabczak *et al.* 1993a,b; Fabczak and Fabczak 1995). These potentials usually appear with a certain response delay in comparison to the onset of light stimulus. The stimulus of high intensity can induce photoreceptor potentials of maximal amplitude in both ciliates which may shift the cell membrane from its resting potential to the electrical threshold for generation of a $Ca^{+2}$ -dependent action potential (Fig. 3).

The elicited action potential, typical for most ciliates, is associated with the observed ciliary beat reversal (phobic response), whereas the photoreceptor potential alone correlates with cell photokinesis, i.e. acceleration of cell swimming. The entire delay in generation of action potential or ciliary reversal in *Stentor* is about 0.5 s and in *Blepharisma* it lasts much longer and takes 2-5 s when measured under the applied conditions (12°C).

The short and intensive stimulus may elicit an action potential of which the repolarising phase is short in time, whereas at prolonged stimulus additional afterpotential depolarisation is displayed. This delayed depolarisation indicates, as it results from the comparison of such membrane response with that including the photoreceptor potential alone, a still maintained photoreceptor potential. The time course of membrane potential changes in dark-adapted cells subjected to long lasting light have a similar pattern as in the case of prolonged stimulus, with the exception that the amplitude of afterpotential depolarisation decreases in time regardless of the continuation of stimulation (Fabczak *et al.* 1993a,b). This observation is possibly related to cell photoadaptation, a phenomenon described in the eye photoreceptor cells of vertebrate or invertebrate organisms (Rayer *et al.* 1990).

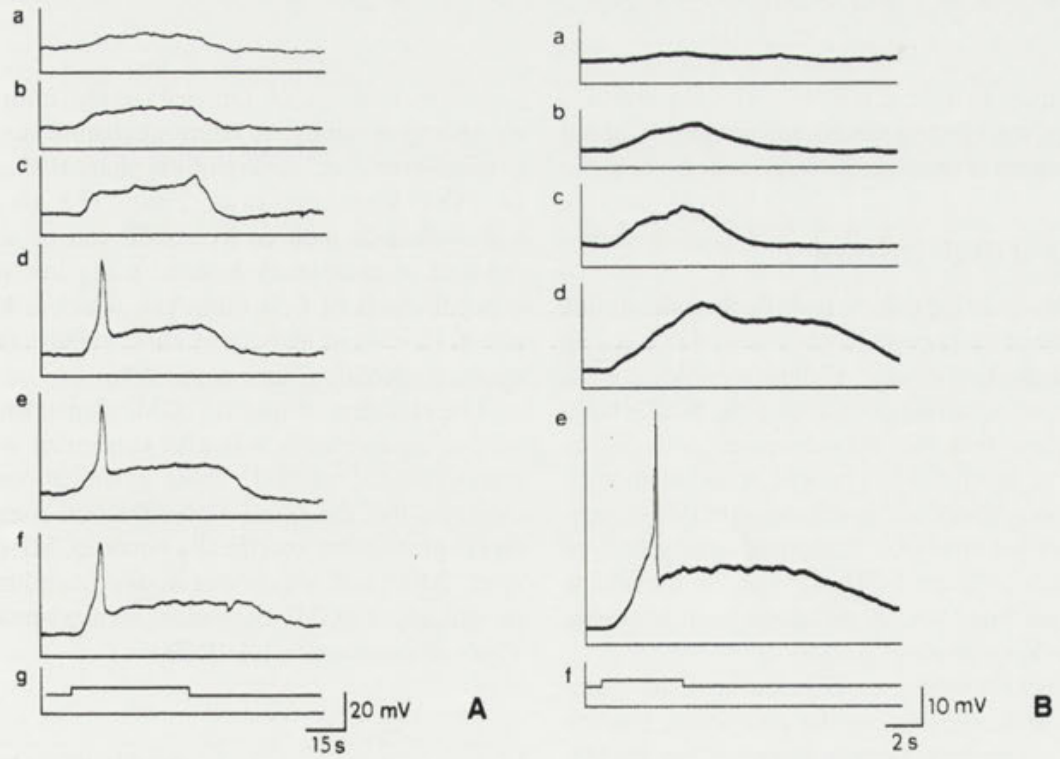



**Fig. 3.** Membrane potential changes in (**A**) *Blepharisma* where in (**a-c**) - photoreceptor potentials are generated alone or in (**d-f**) - generated photoreceptor potentials are followed by action potentials and (**B**) *Stentor* where in (**a-d**) - photoreceptor potentials and in (**e**) - photoreceptor potential and action potential are triggered (from Fabczak and Fabczak 1995)

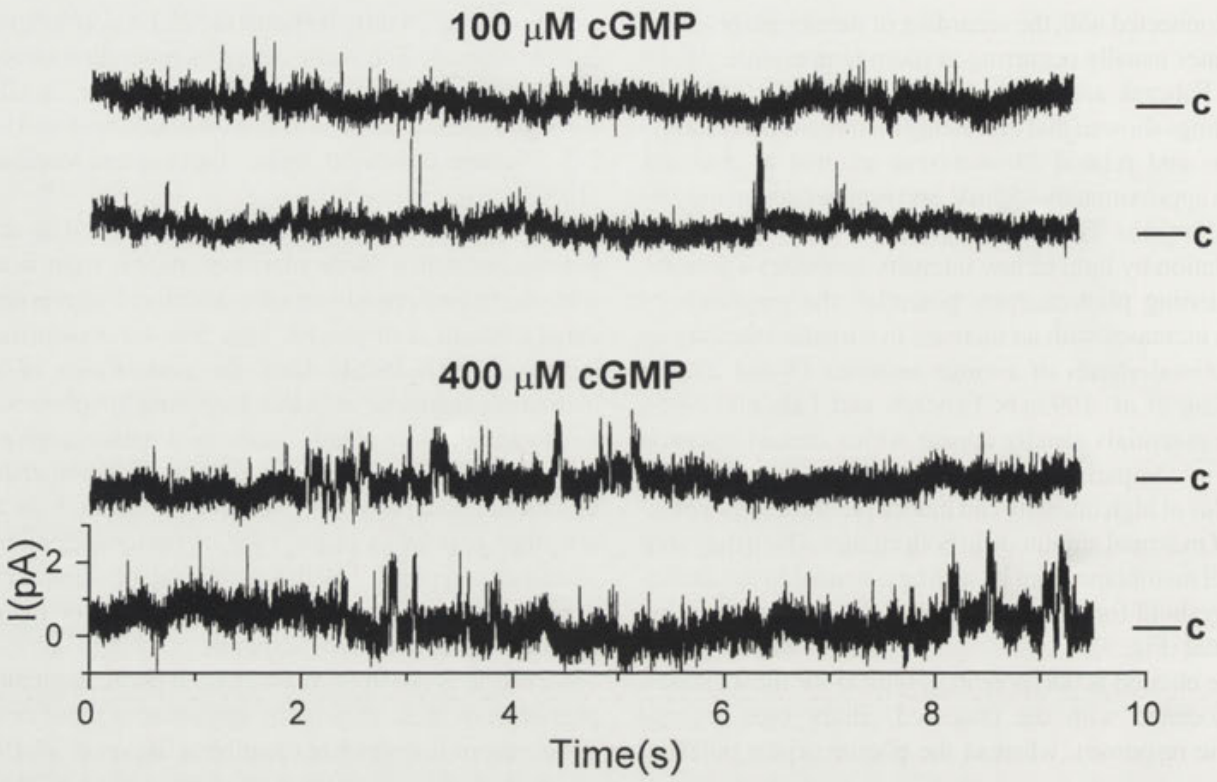



**Fig. 4.** The activity of cGMP-dependent ion channels in native membranes of *Stentor* (from Koprowski *et al.* 1997)

In ciliates adapted to light of high intensity, the photoreceptor potential amplitude is significantly reduced without the ability to generate an action potential and photophobic response.

### Ionic activity of single channels

Based on the existing data, it is difficult to determine the ionic nature of the photoreceptor potential induced by light in photosensitive ciliates. Electrophysiological experiments, recently carried out utilising the patch-clamp method, suggest that the photoreceptor potential in *Stentor* may be generated by changes in cell membrane conductance as a result of the activation cGMP-dependent membrane ion channels. The existence of such type of channels was confirmed (Fig. 4) in native membrane patches excised from vesicles obtained from blistering *Stentor* cells (Koprowski *et al.* 1997).

Similar channels were present in the artificial membrane of liposomes containing the cell cortex fraction from *Stentor*. The ionic conductance of the cGMP-activated single channel in the micromolar cGMP concentration range is about 30 pS (Torre and Menini 1994). The effect of cyclic GMP is fully reversible because complete removal of this nucleotide from the patch environment causes the return of channel activity to the original level, i.e. closing of the channel (Shinozawa *et al.* 1987, Walerczyk *et al.* 2000). The ion activity of these channels induced by cGMP can be significantly reduced or completely blocked using low micromolar concentrations of *l-cis* diltiazem, which is known as a potent blocker of activity of ion channels controlled by cyclic nucleotide (Stern *et al.* 1986).

The existence of specific cGMP-dependent ion channels in *Stentor* cells was also confirmed with an immunochemical method. These examinations evidently indicated that the ciliate cortex fraction comprised of a 63 kD protein that specifically bound cGMP (Walerczyk *et al.* 2000) and which was highly homologous to the α- subunit of cGMP-dependent channel protein in bovine rod outer segments (ROS).

**photokinesis ⇒ photodispersal**
⇑
**light ⇒ photopigment ⇒ ?? ⇒ photoreceptor potential ⇒**
**action potential ⇒ photophobic response ⇒ photodispersal**

## LIGHT SIGNAL PROCESSING

### GTP binding protein

As mentioned above, the light-induced action potential thereby ciliary beating reversal in both *Blepharisma* and *Stentor* cells occurs with significant latency. This is quite a long delay when compared to the observed time responses of both the same ciliates to stimuli of different modality, e.g. mechanical stimulation (Wood 1982, S. Fabczak, unpublished,). The depolarising membrane potentials eliciting ciliary reversal appear within the milliseconds period in most protozoan ciliates (Eckert 1972, Machemer and Eckert 1973). The delayed action potentials in *Blepharisma* and *Stentor* seem to reflect a intracellular signal processing that finally culminates in photoreceptor potential. Superficially, at least these time response characteristics appear analogous to the signal transduction systems of a variety of metazoan receptor cells that involve specific time-limiting biochemical conversions (Millecchia and Mauro 1969, Firestein 1992). The recent data of behavioural experiments in which marked modulation of photophobic response in *Blepharisma* and *Stentor* by fluoroaluminate, cholera and pertussis toxins as well as mastoparan, i.e. the substances that modulate the activity of GTP binding proteins (G-proteins) in a significant way, seem to confirm the above suggestions.

For many years, it has been known that G-proteins are an important step in a process of signal processing in eukaryotic receptor cells. Therefore, the effectiveness of signal processing can be significantly influenced when these modulators are applied. It has been known that fluoroaluminate may activate G-proteins by binding to them in a similar way as GTP (Bigay *et al.* 1982). Cholera and pertussis toxins are known to cause catalytic ADP-ribosylation of transducin, i.e. G-protein involved in signal transduction by photoreceptor cells of vertebrates, and inhibits GTP-ase activity, thus maintaining G-protein in an active state (Watkins *et al.* 1984). Mastoparan is, in turn, a factor which can directly activate G-proteins, thus fulfilling the role of the stimulus itself (Higasijima *et al.* 1990).

In *Stentor* cells, both fluoroaluminate and toxins cause a significant increase of cell sensitivity to light stimulation (Fabczak *et al.* 1993c). Ciliates subjected to the influence of these substances reacted faster to a light stimulus (shorter delay of photophobic response), as well as a greater number of photoresponding cells. A similar effect, i.e. increased photosensitivity, was displayed by *Blepharisma* after preliminary incubation of ciliates in a medium containing fluoroaluminate, both toxins or mastoparan (Fabczak *et al.* 1993d, Fabczak 2000).

The data of experiments using Western blot and PCR further support the possibility of the existence in ciliates of a photoreceptor processing system with the participation of G-proteins. Immunoblot analysis of either cell lysate or cortex fraction of *Stentor* cells with antibodies raised against the α-subunit of transducin reveals a major protein band of 39 kD, which is similar in apparent molecular mass to the transducin from photoreceptor cell of bovine retina. The highly sensitive response with the used antibodies evidently indicate that the 39 kD protein found in *Stentor* implies it is at least partially homologous to the α-subunit of transducin. Amino acid sequence alignment data also indicate that the protein is homologous to about 35% with the α-subunit of different heterotrimeric G-proteins existing in other eukaryotic organisms (Fabczak *et al.* 1993 d, Song 1997b). In the related *Blepharisma,* identical examinations showed the presence in the cell lysate or cortex fraction of a 55 kD protein, responding with antibodies produced against both the α-subunit of transducin and common fragment of the α-subunit of G-proteins (Fabczak 2000). Among other investigated ciliates, the existence of heterotrimeric G-proteins were shown in another photosensitive ciliate, *Paramecium bursaria*. This ciliate possesses a protein of molecular mass of 57 kD, which shows GTP-ase activity and is also homologous to the α-subunit of G-proteins (Shinozawa *et al.*1996, New and Wong 1998).

**photokinesis ⇒ photodispersal**
⇑
**light ⇒ pigment ⇒ G-proteins ⇒ ? ⇒ photoreceptor potential ⇒ action potential ⇒ photophobic response ⇒ photodispersal**

### Cyclic GMP and trisphosphoinositol as second messengers

The first suggestion for secondary messenger participation in the phototransduction pathway in ciliates comes from studies on the elongation of the *Blepharisma* cell body when it is exposed to prolonged light of high intensity (Ishida *et al.*1989). It was shown in these experiments that light-induced cell elongation is mediated by changes in internal cyclic nucleotide concentrations and is inhibited in the presence of mononucleotide

phosphodiesterase antagonists or the membrane permeable analogue of cGMP.

Cyclic GMP also seems to be engaged in the cell photophobic responses of both *Blepharisma* and *Stentor* (Fabczak *et al.* 1993c,d). It has been found recently that membrane permeable analogues of cGMP, 8-bromo-cyclic GMP (8-Br-cGMP) or dibutyryl-cGMP, specifically suppress the photophobic responses in these ciliates as there was no significant changes in the photomotility in ciliates exposed to the membrane permeable analogues of cyclic AMP (8-Br-cAMP and dibutyryl cAMP) under identical conditions (Fabczak *et al.* 1993c,d; Walerczyk *et al.* 2000). This specific inhibitory influence on the cell photobehavior is entirely reversible and depends on both the duration of incubation and the concentration of the nucleotide used. A similar effect on the cell photophobic responses was observed when *Blepharisma* and *Stentor* were treated with compounds, known widely to increase the level of cGMP in the cells such as inhibitors of cyclic nucleotide phosphodiesterase (PDE), 3'-isobuthyl-methylxanthine (IBMX) or theophylline. They increase the level of cGMP in the cell cytoplasm and cause a significant increase in the latency of the photophobic response and decrease in the number of photoresponsive cells. In contrast, the G-protein activators, fluoroaluminate and 6-anilino-5, 8-quinolinedione (LY83583), which lower cellular cGMP levels in a range of tissues (Schmitt *et al.* 1985, O'Donnell and Owen 1986), cause a marked increase of ciliate photoresponiveness (Fabczak *et al.* 1993c,d; Walerczyk *et al.* 2000).

Using a specific radioimmunoassay to investigate the intracellular cGMP levels in *Stentor*, further data were obtained testifying to the involvement of cGMP in the cell light signal transduction mechanism (Walerczyk *et al.* 2000). In cells adapted to darkness and then subjected to light stimulation, a fast decrease in the level of cytoplasmic cGMP occurs, followed by its slower increase to the basal value. These transient alterations in the level of cGMP are dependent on the intensity and duration of the light stimuli used. The light-dependent changes in intracellular cGMP level of *Stentor* were greatly reduced in the case of cells preincubated with IBMX and theophylline. Preliminary experiments connected with assessing, in an identical way, the level of intracellular cGMP in related ciliate *Blepharisma* did not afford convincing data on the role of cGMP in the photoresponse mechanism in this ciliate (H. Fabczak, unpublished data).

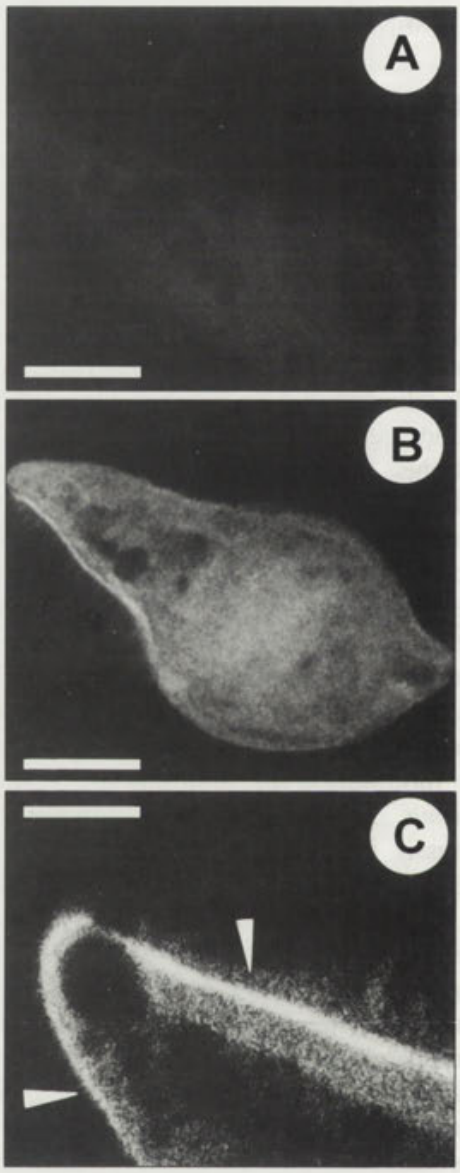


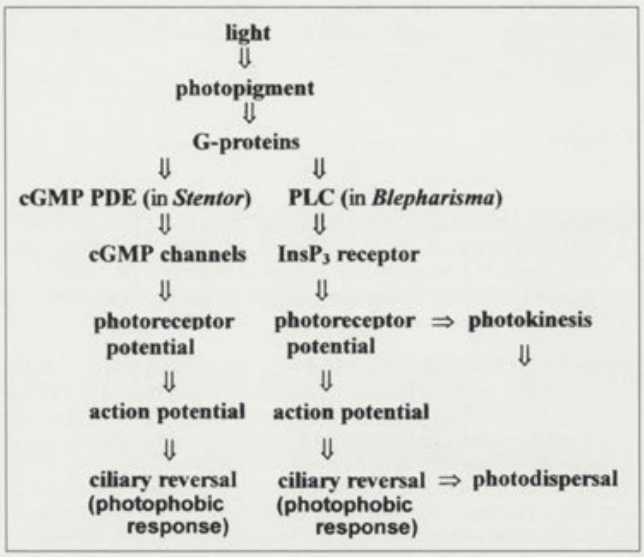

**Fig. 5.** Identification of protein homologous to $InsP_3$ - receptor in *Blepharisma* cells: (**A**) - control cell preincubated without primary antibody), (**B**) and (**C**) - cells incubated with primary antibody against $InsP_3$- receptor and secondary antibody fluorescein conjugate (from Fabczak *et al.* 1998). Scale bars - **A**, **B** - 20 μm; **C** - 60 μm

Earlier behavioural examinations utilising agents known to modify the phosphoinositol signalling pathway in various cells suggested, however, that the photophobic response in *Blepharisma* may be mediated by another second messenger, lipid-derived inositol 1,4,5-trisphosphate ($InsP_3$). This stems from observations that neomycin, a well known inhibitor of the hydrolysis of phosphatidylinositol to $InsP_3$ and diacylglycerol (DAG)

(Gabev *et al.* 1989, McDonald and Mamrack 1995), elicits a significant decrease in cells photosensitivity (Fabczak *et al.* 1996, Fabczak 2000). Similar changes in cells photobehaviour also occurs under the influence of heparin, a blocker of $InsP_3$ receptor (Supattapone *et al.* 1988) and lithium ions, used to inhibit activity of monophosphoinositol phosphatase and to decrease the amount of $InsP_3$ (Berridge 1987, Nahorski *et al.* 1991, O'Day and Phillips 1991).

The analysis of the level of $InsP_3$ with the use of a specific radioimmunological method showed that light undoubtedly causes a temporary increase in the level of $InsP_3$ in *Blepharisma* cells during illumination. The light-induced levels of internal $InsP_3$ in the ciliate increase with increasing stimulus intensity and are significantly inhibited in ciliates preincubated with $Li^+$ or neomycin (Fabczak *et al.* 1999). A performed analysis of the cell cortex fraction of *Blepharisma* cells with a Western blot method unequivocally showed the existence, in the cortex fraction, of a protein of molecular mass greater than 200 kD, homologous to the $InsP_3$ receptor protein from receptor cells of higher organisms. The presence of this $InsP_3$ -receptor like protein in the cell cortex layer was additionally confirmed by the results of immunocytochemical experiments (Fabczak *et al.* 1998) using antibodies marked with fluorescein (Fig. 5). Recently it has been reported by Matsuoka *et al.* (2000) that novel photoreceptor blepharismin-200 kD protein complex found in *Blepharisma* is possibly related to $InsP_3$-like protein. An introduction of antisense oligonucleotide for $InsP_3$ receptor or anti- $InsP_3$ receptor antibody into living cells of *Blepharisma* caused decrease in cell photosensitivity and reduction of content of blepharismin-200 kD protein.

In light of the above mentioned data, it seems that cGMP and/or $InsP_3$ may be involved in the light signal transduction pathways of *Blepharisma* that lead to changes in membrane potentials and the photophobic response in analogy to some photoreceptor cells of invertebrates (Rayer *et al.* 1990, Gotov and Nishi 1991, Dorlöchter and Sieve 1997). It cannot be excluded that both pathways interact with each other and cGMP can be used by the cell, for example in a later phase of signal transduction to regulate the activity of $InsP_3$ receptor though its phosphorylation with participation of a cGMP-dependent kinase (Komalavilas and Lincoln 1994a,b).




## CONCLUDING REMARKS

The results of the studies described above are attempts to explain the complicated mechanism of light signal transduction in two closely related ciliates *Blepharisma* and *Stentor*. Some steps of the mechanism are still unknown and require further detailed studies. So far there is no data regarding the functional activity of cellular photoreceptors, blepharismin and stentorin, *in vitro*. From some experiments already carried out, it seems likely that these pigments can irreversibly lose their activity during isolating procedures from the remaining protein structures that create the supposed path of signal processing (Song 1997a,b). In *Blepharisma* and *Stentor*, further investigations are also needed for the nature of the ionic mechanism of photoreceptor potential, the final step in the process of light signal processing system, to be elucidated.

It seems, as mentioned above, that the generation of photoreceptor potential in *Stentor* is mediated by the activity of membrane ion channels, which may be controlled by secondary messengers, though there also exists in literature other hypotheses on this subject (Wood 1991). Particularly interesting is the fact that regardless of the similarity in electrical and motile responses of closely related *Blepharisma* and *Stentor*, different second messengers seem to be involved (see diagram above) in some steps of light signal processing.

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Review Article

# Facilitated Hexose Diffusion in Kinetoplastida

**Leszek SZABLEWSKI**

**Department of General Biology and Parasitology, Center of Biostructure, Medical University of Warsaw, Warszawa, Poland**

**Summary.** The kinetoplastid glucose transporters belong to the glucose transporter superfamily, exemplified by the mammalian transporters, especially GLUT1. Some species, which undergo a life cycle in which parasitic stages are exposed to different glucose concentrations in several hosts, have evolved two different transporters to deal with this difference. While all of the trypanosome transporters also carry D-fructose, GLUTs (excluding GLUT2 and GLUT5) do not. Mammalian glucose transporters are very much more susceptible to cytochalasin B and phloretin, inhibitors of GLUTs, than are the trypanosome transporters. These properties suggest that the glucose transporter may be a good target for anti-trypanosomal drugs. The trypanosome hexose transporter might also be a vaccine candidate if it is accessible to antibodies. Genes encoding proteins involved in glucose transport have been cloned from several kinetoplastid species. Typically, the expression of hexose transporter genes is stage-regulated. The putative hexose transporter genes are highly conserved among Kinetoplastidae.

**Key words:** facilitated diffusion, gene expression, gene organization, hexose transporters, Kinetoplastida.

## INTRODUCTION

Carbohydrates, (glucose in particular), are an important source of energy for most living organisms. However, as the lipid bilayers that make up cell membranes are impermeable to carbohydrates, carbohydrate-transport systems are required. In recent years, it has proved possible to clone two distinct molecular families of cellular transporters of glucose and other hexoses.

Address for correspondence: Leszek Szablewski, Department of General Biology and Parasitology, Center of Biostructure, Medical University of Warsaw, ul. Chałubinskiego 5, 02-004 Warszawa, Poland; Tel/Fax: 628-53-50; E-mail: lszabl@ib.amwaw.edu.pl

Cotransporters are a major class of membrane proteins that are formed by members of several gene families. They share the common property of being able to couple the electrochemical potential gradient of a cation to transport organic solutes, ions, and water „uphill" into cells. This type of transport system has been described in many organisms and includes, for example, $Na^+$/coupled glucose transporters (SGLTs) in mammals (Loo *et al.* 1998, Rhoads *et al.* 1998, O'Connor and Diamond 1999). Over 35 members of the SGLT1 family have been identified from animal cells, yeast and bacteria (Turk and Wright 1997, Wright *et al.* 1998).

The other group of transporters conveys glucose by facilitated diffusion down glucose-concentration gradients. This group consists of seven homologous trans-

membrane proteins, GLUTs1-5, GLUT7 and GLUTX1 that are encoded by distinct genes. These proteins are also widely distributed in organisms (Pessin and Bell 1992, Gould and Holman 1993, Mueckler 1994, Sa-Noguiera and Ramos 1997, Szablewski 1998, Szablewski *et al.* 1999, Ibberson *et al.* 2000). Glucose transporter genes have been cloned from several parasitic protozoa of the order *Kinetoplastida* (Snapp and Landfear 1997, Tetaud *et al.* 1997), and have been expressed functionally in *Xenopus* oocytes and/or Chinese hamster ovary (CHO) cells (Barrett *et al.* 1998).

The order *Kinetoplastida* comprises unicellular flagellates. Several species are important parasites, for example causing sleeping sickness in humans and a number of veterinary diseases, as in the case of African trypanosomes. *Leishmania* species causes a wide spectrum of disease world-wide, while *Crithidia fasciulata* is known as a parasite of insects

*Kinetoplastida* are typically parasites with a digenetic life cycle; however a few, for example *Trypanosoma equiperdum,* live in one host only. Amastigotes of *Leishmania* are forms of the parasite that live within the macrophages of the mammalian host. The other life cycle form, the promastigote, is specialized for the colonization of the insect alimentary tract. The same is true of African trypanosomes, which differentiate into several adaptative forms, the most prominent of which are the bloodstream form in the mammalian host and the procyclic form in the midgut of the tsetse fly vector. The great difference between these hosts has required the forms to adapt to differing environmental conditions. For example, they differ in their metabolism (Fairlamb and Opperdoes 1986). The availability of free glucose in serum differs greatly from that in a mammalian cells' cytoplasm or within the midgut of insect vectors. All kinetoplastid species have specific plasma membrane transporters to facilitate the uptake of hexose. On the other hand infective bloodstream forms use glucose only as a carbon source, while the procyclic stage from the insect midgut can thrive in the absence of glucose, preferring amino acids (mainly proline) as an energy source (Parsons and Nielsen 1990, Ter Kuile and Opperdoes 1991). These characteristics suggest that glucose transport might be stage-regulated.

There are currently no satisfactory drugs to use against these parasites, and no vaccines exist. It is likely that the glucose transport system might provide an alternative target for chemotherapy, as might gateways that allow for the targeting of other toxic molecules of these parasites. Moreover, such transporters represent potential targets for immunotherapy.

## THE STRUCTURE OF GLUCOSE TRANSPORTERS

In 1985, Mueckler *et al.* cloned and sequenced a gene encoding a human facilitated glucose transporter (GLUT1). Analysis of the sequence using a hydrophobicity profile program (Kyte and Doolittle 1982) led to a proposed model for the secondary structure. According to these authors, the transporter is composed of twelve putative hydrophobic transmembrane segments separated by hydrophilic loops. The N and C termini are located on the cytoplasmic side of the plasma membrane. Two large hydrophilic loops, one external between the putative transmembrane domains 1 and 2, and the other internal between transmembrane helices 6 and 7, were found. An extracellular loop of 33 amino acids (the first loop) is the location of a single asparagine (Asn) linked oligosaccharide addition.

In the case of the THT (for *Trypanosoma* Hexose Transporter), the hydropathy plot of the sequence, determined by the above mentioned method, is similar to that of the human glucose transporter (Bringaud and Baltz 1992). The THT contains hydrophilic N and C termini. The largest extracellular loop in the THT resembles the LTP (for *Leishmania* Transporter Protein [Cairns *et al.* 1989, subsequently designated Pro1 (Langford *et al.* 1992)], in containing a large number of cysteines (6 over 50 amino acids and 8 over 83, respectively) while the loop between transmembrane domains 6 and 7 is relatively small (Tetaud *et al.* 1997). The cysteines are probably involved in disulfide bonds. This conformation is consistent with a stable folded structure resistant to protease digestion - a property that may be important in the biological functioning of this molecule in promastigote forms found in the midgut of insects (Bringaud and Baltz 1992). These cysteine-rich protein segments are not found in the other known sugar transporters.

Amino acid analysis of the various kinetoplastid glucose transporters has revealed significant differences in the occurrence of potential N-linked glycosylation sites (Barrett *et al.* 1998). Asn-69 in *T. brucei* THT2, Asn-81 in *T. cruzi* TcrHT1, and Asn-90, and Asn-91 in *T. vivax* TvHT1 are the only potential exofacial N-glycosylation sites in these transporters, all located within the first extracellular loop.

The *T. brucei* bloodstream form transporter, THT1, lacks potential N-linked glycosylation sites on any of the predicted extracellular loops. THT1 does contain one N-glycosylation consensus sequence (Asn-7), although this is located on the amino terminal tail and is predicted to face the cytoplasm. THT2 (Asn-7) and TvHT1 (Asn-10) also have an additional N-glycosylation consensus sequence at similar positions (Barrett *et al.* 1998). In *Leishmania* spp., no potential N-linked glycosylation site was identified in the first extracellular loop, so characteristic for GLUTs.

The different kinetoplastid glucose transporter amino acid sequences are all highly homologous with one another. Alignments of the amino acid sequences of the different transporters from *T. brucei* (THT1 and THT2), *Leishmania* (Pro1-Iso-1, Pro1- Iso-2 and D2), *T. cruzi* (TcrHT1) and *T. vivax* (TvHT1) reveal the great similarity of all of these proteins (30-85% similarity, with most conservation in the central part and towards the C-terminus of the protein) (Tetaud *et al.* 1997). For example, comparison of the sequence of amino acids in the THT with the sequence of Iso-2 revealed 46.5% identity between the two proteins, and a level of 68.3% similarity when conservative amino acid substitutions were considered (Bringaud and Baltz 1992). The *L. donovani* D2 transporter is the most divergent, suggesting a distinct physiological role consistent with its extremely high $K_m$ for D-glucose (Langford *et al.* 1995).

When compared with GLUT1, THT has almost the same percentage identity (19.2%) and similarity (42.5%) as LTP has with GLUT1 (21.7% and 44.4%, respectively) (Cairns *et al.* 1989). In addition, comparison of the THT sequence with other sugar transporters, i.e. arabinose/$H^+$ and xylose/$H^+$ transporters from *Escherichia coli* (Maiden *et al.* 1987), the hexose carrier from *Chlorella* (Sauer and Tanner 1989), the yeast SNF3 glucose transporter (Celenza *et al.* 1988) and the yeast GAL2 galactose transporter (Nehling *et al.* 1989), indicates the presence of 15 amino acids strictly conserved in their specific positions (Bringaud and Baltz 1992). These findings are consistent with the notion that these residues serve critical and related functions in all of these proteins (Cairns *et al.* 1989).

There are several blocks of sequences conserved between the THT and GLUT1. A first block consisting of QLTGINAV (315-322) is also present in LTP (Cairns *et al.* 1989) and two blocks VGSMVGS (130-136) and PMYVNE (197-202) are more specific to THT and GLUT (Mueckler *et al.* 1985). These three blocks of sequences are also very conserved in the five other sugar transporters mentioned above (Nehling *et al.* 1989, Sauer and Tanner 1989). The other sequences of the GLUT (residues 89-93 and 330- 334), which are conserved in many known transporters and are only moderately conserved at the same relative position in LTP, are not present in THT. Similarly, several highly-conserved regions of many of the known sugar transporters are not conserved in the THT, most notably PESPR and PETKG (residues 208-212 and 454-458 of GLUT) (Bringaud and Baltz 1992). The three consensus ATP-binding sequences found in GLUT (Carruthers and Helgerson 1989) and partly conserved in the other mammalian glucose transporters are also not present, at least not in the same relative position, in the THT (Bringaud and Baltz 1992).

Arginine is shown to play a role in the interaction of transporters with substrate (Tetaud *et al.* 1996). A comparison of the different sequences of kinetoplastid hexose transporters (Pro-1, D2, THT1, THT2, TcrHT1 and TvHT1) reveals four conserved arginine residues. The residues are located in transmembrane segment 4 and between helices 5/6, 8/9 and 10/11. Three of these residues (those located in transmembrane helix 4 and between transmembrane helices 8/9 and 10/11) are highly conserved in other members of the glucose transporter superfamily (Baldwin 1993), and one or more of these may be critical in substrate binding (Tetaud *et al.* 1997). The arginine residue located in the loop between transmembrane domains 8/9 of the GLUT4 plays a direct role in glucose uptake (Wandel *et al.* 1995).

Differential subcellular localization has been noted for the *Leishmania* transporters. Pro1-Iso-1 is found principally in the flagellar membrane, while Pro1-Iso-2 occurs in the plasma membrane and flagellar pocket. The different N-terminal sequences may target the different isoforms (Piper *et al.* 1995). The 130 amino acid $NH_2$-terminal cytoplasmic domain of the isoform 1 glucose transporter is sufficient to target a non-flagellar integral membrane protein into the flagellar membrane (Snapp and Landfear 1999).

An essential flagellar targeting signal is located between amino acids 20 and 35 of the N-terminal sequence (Snapp and Landfear 1999). The Iso-2 associates with the microtubular cytoskeleton that underlies the cell body membrane. The second isoform (Iso-1, flagellar membrane isoform) does not associate with the cytoskeleton (Snapp and Landfear 1997). These transporters are struc-

turally similar to other members of the glucose transporter superfamily, and can be expressed in heterologous systems.

## GENOMIC ORGANIZATION OF THE HEXOSE TRANSPORTERS

Glucose transporter genes from several kinetoplastids have been cloned and expressed functionally in *Xenopus* oocytes and/or CHO cells (Cairns *et al.* 1989, Tetaud *et al.* 1997). Some kinetoplastids contain a multigenic family encoding two isoforms of glucose transporters (Stack *et al.* 1990, Barrett *et al.* 1998, Bringaud *et al.* 1998). In contrast, there are also species which express a single isoform encoded by tandemly-repeated genes (Bringaud and Baltz 1992, Bringaud and Baltz 1993, Tetaud *et al.* 1994, Waitumbi *et al.* 1996). Some of these glucose transporters are regulated developmentally. The results suggest that kinetoplastids have a high level of conservation in gene organization (Bringaud *et al.* 1998), indicating an important role for these proteins in the parasite life cycle.

*Leishmania enriettii* contains the gene Pro-1 encoded LTP (for *Leishmania* Transporter Protein) (Cairns *et al.* 1989). Detailed analysis of the 5' repeat units has revealed the presence of two isoforms (Iso-1 and Iso-2), which differ only in the size, and sequence of the N-termini (Stack *et al.* 1990). Partial sequence analysis reveals the presence of one Iso-1 copy followed by eight Iso-2 copies (Stein *et al.* 1990). This gene is regulated developmentally. The mRNA from Pro-1 accumulates to a much higher level in the promastigote stage of the parasite life cycle in the gut of the insect than in the amastigote stage of the parasite that lives inside the macrophage of mammalian cells (Cairns *et al.* 1989).

Similar organization was found in *L. donovani*. Analysis of the obtained results revealed the presence of one Iso-1 copy followed by four Iso-2 copies (Bringaud *et al.* 1998). Two further genes, called D1 and D2, with identity to the glucose transporter family were also cloned from *L. donovani* (Langford *et al.* 1992). Both genes are present as single copies. D2 is very similar to Pro-1. In contrast, D1 is structurally quite different from either D2 or Pro-1 and is more similar in sequence to the mammalian transporter GLUT1. The functional expression of the D1 gene in *Xenopus* oocytes revealed it to encode a plasma membrane myo-inositol/$H^+$ symporter rather than a hexose transporter (Drew *et al.* 1995). Both D2 and Pro-1 are developmentally-regulated genes, which are expressed, primarily in the insect stage of the parasite life cycle, when the concentration of sugar reaches very high levels and so a high $K_m$ would be advantageous (Langford *et al.* 1995). In contrast, D1 is not regulated during the parasite life cycle. All three genes are located on different chromosomes in *L. donovani* (Langford *et al.* 1992).

The five species of Salivarian trypanosomes (also called African trypanosomes: subgenus *Trypanozoon* or *Trypanosoma brucei* group) i.e. *Trypanosoma brucei brucei, T. b. gambiense, T. b. rhodesiense, T. equiperdum* and *T. evansi*, contain a multigenic family encoding two isoforms of glucose transporters referred to as THT1 and THT2 (for *Trypanosoma* Hexose Transporter). These two isoforms are 89% identical (Bringaud and Baltz 1992, Bringaud and Baltz 1993). Genes are arranged in a head to tail fashion with a cluster of THT2 genes following a cluster of THT1 genes with copy number varying between strains. For example, *T. brucei* contains six copies of THT1 and five copies of THT2 (Bringaud and Baltz 1994, Barrett *et al.* 1996), while in *T. congolense* TcoHT1 and TcoHT2 genes alternate (Bringaud *et al.* 1998). Analysis of the polymorphism in gene-copy number for both isoforms in numerous strains has revealed them to be present in multiple copies in tandem arrays, with copy number varying in a strain-specific manner.

These genes are regulated developmentally. In *T. brucei* the THT1 isoform is a low-affinity transporter and is expressed in bloodstream forms (40-fold more THT1 than THT2), whereas procyclics express THT2 (high-affinity form), but no detectable (or very low levels of) THT1 mRNA, depending on the strain. The bloodstream forms contain about 40-times more stable mRNA encoding THT1 than THT2 (Bringaud and Baltz 1993). In contrast, *T. vivax* and *T. cruzi* express a single isoform (TvHT1 and TcrHT1, respectively) encoded by tandemly repeated genes (Tetaud *et al.* 1994, Waitumbi *et al.* 1996).

The differential expression of kinetoplastid stage-regulated genes is well documented (Borst 1986, Gibson *et al.* 1988, Wirtz *et al.* 1991), but there are few examples of genes whose expression is regulated by specific environmental agents. The parasites therefore have two transporter genes that are expressed in a fashion allowing maximal exploitation of the host's extracellular environment. Bloodstream forms express predominantly a high-capacity, low-affinity transporter to exploit the high con-

centration of glucose in mammalian serum. Procyclic forms of *Trypanosoma* express the higher-affinity transporter in the insect midgut where glucose is relatively scarce, and amino acids become the major energy source (Barrett *et al.* 1998). For example, a $K_m$ of the THT1 for D-glucose of 0.053 mM was reported (Tetaud *et al.* 1997). Other species, for example *T. cruzi*, have a single glucose transporter gene isoform, which is expressed at similar levels in epimastigotes, which live in the insect midgut, and trypomastigotes which live transiently in the bloodstream of mammals (Barrett *et al.* 1998). Interestingly, *T. cruzi* express a high-affinity glucose transporter, consistent with the fact that at least part of its life cycle occurs in the low glucose environment of the cell interior (Barrett *et al.* 1998). A $K_m$ of TcrHT1 for D-glucose of 0.08-0.3 mM has been reported (Tetaud *et al.* 1997).

## THERAPEUTIC TARGETS

Currently there are no satisfactory drugs for use against described parasites, and no vaccines exist. The mode of action of many reagents is unknown, and currently-used compounds were derived empirically. To increase the chances of success, the development of new drugs should be aimed at those steps in the metabolic pathway, which are either absent, or differ from analogous steps in the host. In the case of trypanosome glucose transporters significant differences can be identified in terms of both the pharmacology and substrate recognition profiles, when compared to the GLUT. All of the trypanosome glucose transporters also recognise D-fructose, which distinguishes them from the main mammalian glucose transporter, GLUT1 (Barrett *et al.* 1998). However, at least 6 other mammalian plasma membrane hexose transporters are expressed in different tissues, with a range of substrate specificities, including two isoforms (GLUT2 and GLUT5) (Burant *et al.* 1992, Colville *et al.* 1993) which also recognise D-fructose, high-lighting the difficulties in pinpointing unique features and compromising their utility as chemotherapeutic targets. However, differences between mammalian and kinetoplastid hexose transporters have been described. For example, GLUT1 transports D-fructose with 1000-fold less efficiency than D-glucose (Eisenthal *et al.* 1989) and is 93% inhibited by 5 μM cytochalasin B (Kasahara and Hinkle 1977), while the THT1 transports fructose and is only 53% inhibited by 300 μM cytochalasin B (Bringaud and Baltz 1993). All of the kinetoplastid hexose transporters are also relatively insensitive to the classical inhibitor of GLUT1 transport, phloretin (Tetaud *et al.* 1997).

The *T. brucei* bloodstream form transporter does not make hydrogen bonds with the hydroxyls at positions 2 and 6 of the glucose ring (Eisenthal *et al.* 1989). These sites are considered available for the attachment of other chemical constituents, which would not interfere with recognition by the transporter. In the case of the C-6 position, a strict limit on the size of substituent groups has been noted (Barrett *et al.* 1998). Relatively large replacements could be added at position C-2. At least one compound containing a substituent group at position C-2 has been developed, being toxic to bloodstream form parasites grown *in vitro* (Barrett *et al.* 1998). Fructose analogues have also been developed, and toxic examples are known (Page *et al.* 1996).

In the case of *T. cruzi*, the glucose transporter does not recognize C-3 or C-6 analogues of D-glucose. Glucose molecules substituted at C-6, might therefore be useful in the treatment of African sleeping sickness, but unfortunately not Chagas' disease (Tetaud *et al.* 1996). The capacity of these parasite transporters to transport D-fructose with a high affinity compared with mammalian hexose transporters may represent a more useful means of developing toxic molecules specific for Kinetoplastida (Fry *et al.* 1993).

As surface molecules, the transporters may be immunogenic and exposed to the immune system. The parasite and host glucose transporters have important amino acid differences in their exofacial loops. The immunogenicity of the specific epitopes of these glucose transporters will be tested to determine potentials as eventual vaccines (Bringaud and Baltz 1993, Tetaud *et al.* 1997).

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PROTOZOOLOGICA

# Human Chorionic Gonadotropin (HCG)-like Hormones (FSH, LH, TSH) in *Tetrahymena*. A Confocal Microscopic Analysis

**György CSABA and Péter KOVÁCS**

**Department of Genetics, Cell and Immunobiology, Semmelweis University, Budapest, Hungary**

**Summary.** The unicellular ciliate, *Tetrahymena* contains molecules which immunologically and functionally resemble to human hormones. Up to now, of the pituitary glycoprotein hormones only the presence of adrenocorticotropic hormone (ACTH) was demonstrated. In the present experiment we observed the presence of molecules immunologically similar to human chorionic gonadotropin (HCG). This hormone-like material is localized diffusely in the cell, and in higher amount in the cortical region, in the cilia and in the oral field. After one hour treatment (hormonal imprinting) with luteinizing hormone (LH), follicle stimulating hormone, mixture of these two hormones, HCG, or thyrotropic hormone (TSH), more fluorescence intensity (given by the hormone) can be observed. Twenty four hours or four days after these treatments the amount of hormone-like materials seems to be higher, than in the untreated cells or immediately after treatment. The diffuse localization disappears in the body of the cell after LH or FSH treatment and the hormone concentrates peripheral, in the cortex and especially in (or on) the cilia. The largest amount of hormone-like material seems to be present after LH+FSH or TSH treatment. These results mean that in *Tetrahymena* enhanced production of the appropriate hormone could be induced by hormone treatment (imprinting), which is maintained in the progeny generations. The experiments support that hormonal imprinting of receptor and hormone production can run parallel.

**Key words:** gonadotropins, hormonal imprinting, pituitary hormones, *Tetrahymena*, TSH.

## INTRODUCTION

Unicellular animals, e.g. *Tetrahymena pyriformis* can recognize hormones of higher animals (e.g. mammals) and can react to them (Csaba 1985, 1994). The binding sites for hormones are present in the plasma membrane of the cell and these structures are able to transmit the information given by the hormone to the cell body provoking the reaction of the cell (Csaba 1980). For this aim the protozoan has a transduction system and second messengers, which are similar to those of the mammalian ones (Kuno *et al.* 1979, Kovács *et al.* 1989, Kovács and Csaba 1990, Köhidai *et al.* 1992). In addition, these cells produce and secrete hormone-like materials, immunologically and functionally similar to the hormones of mammals (LeRoith *et al.* 1980,1982,1983; Roth *et al.* 1982). In the case of the first encounter of the *Tetrahymena* and the hormone a special phenomenon, named hormonal imprinting, takes place, which transforms the binding site to real receptor, changing (mainly increasing) and specifying its binding capacity and response (Csaba 1980,1985,1994).

Address for correspondence: György Csaba, Department of Genetics, Cell and Immunobiology, Semmelweis University, Nagyvárad tér 4, POB 370, 1445 Budapest, Hungary; E-mail: csagyor@net.sote.hu

The effect of hormonal imprinting is transmitted to hundreds of progeny generations (Csaba *et al.* 1982 a), helping the recognition capacity of the cells and their chance for life by it (Csaba 2000).

The hormonal imprinting evokes not only receptor development, but provokes the enhanced production of the imprinter hormone. Insulin for example is taken up by *Tetrahymena* and after many generations its level is higher in the treated cells, than in the controls (Csaba and Kovács 1995, Csaba *et al.* 1999). A similar phenomenon was observed in the case of endorphin (Csaba and Kovács 1999). These facts could be explained by Blalock's molecular recognition theory (Blalock and Smith 1984, Bost and Blalock 1989), which hypothesized that interacting peptides are coded by the complementary DNA strands.

Hormones of the anterior pituitary and peptide hormones of the placenta belong to the same hormone family having identical α-subunits and similar β-subunits (Frieden 1976). This makes possible the study of the selection capacity of *Tetrahymena* between related hormones. To this end, in the present experiments the presence of HCG-like hormones and the effect of different hormones of the above mentioned family on hormone production are studied in *Tetrahymena*, using antibody to HCG and confocal microscopy.

## MATERIALS AND METHODS

*Tetrahymena pyriformis* GL strain was used in the logarithmic phase of growth. The cells were cultured at 28 °C in tryptone medium (Sigma, St. Louis, USA) containing 0.1% yeast extract. The density of *Tetrahymena* cultures was $10^4$ cell/ml.

### Treatment of cultures and collecting of samples

Cultures were treated with $10^{-6}$ M (this is the usual hormone concentration for imprinting) human chorionic gonadotropin (HCG, Choriogonin, Richter, Budapest); or with a mixture of follicle stimulating hormone and luteinizing hormone (FSH+LH, Pergonal, Human Gödöllő, lic. Serono, Italy); or with FSH (Metrodin, Serono) or thyrotropic hormone (TSH, Ambinon, Organon, Oss) for 1 h. In the control groups, the hormone vehicle alone was added. Samples were taken immediately after treatment and other portions of the cultures transferred to fresh medium every second day. Samples were taken again 24 and 96 h after treatment.

### Immunocytochemical localization of HCG-like materials

After washings, the cells were fixed with 4% paraformaldehyde solution (dissolved in pH 7.2 PBS) for 5 min, and washed twice in wash buffer [0.1% BSA; 20 mM Tris-HCl; 0.9% NaCl; 0.05% Tween 20 (as detergent); pH 8.2]. To block non-specific binding of antibodies, the cells were treated with 1% BSA in PBS for 30 min at room temperature. Aliquots from cell suspensions (50 µl) were transferred to Eppendorf microfuge tubes, and 50 µl primary antibody (rabbit anti-HCG from Sigma, St. Louis, Mo. USA) diluted 1:200 in antibody buffer (1% BSA in wash buffer) was added for 45 min at room temperature. The antibody was specific to rat and human chorionic gonadotropin, with 20%, 5.6%, 2%, and <0.1% cross-reactivity to human luteinizing hormone, thyroid stimulating hormone, follicle stimulating hormone, and growth hormone, respectively. Negative controls were carried out with 50 µl PBS containing 10 mg/ml BSA instead of primary antibody.

After washing 4 times with wash buffer to remove excess primary antibody, the cells were incubated with secondary antibody (FITC-labeled monoclonal goat anti-rabbit IgG (Sigma), diluted 1: 50 with antibody buffer for 30 min at room temperature. After 4 further washings with buffer, the cells were mounted onto microscopic slides.

The labeled and mounted cells were analyzed in a BioRad MRC 1024 confocal laser scanning microscope, equipped with a krypton-argon mixed gas-laser as a light source, at an excitation wavelength of 480 nm line. All experiments were repeated twice.

## RESULTS

In the control *Tetrahymena* with complete immunocytochemistry (1st and 2nd antibody) there is a diffuse fluorescence, which is enhanced in the cortical region, oral field and cilia (Figs. 1B, C). The most prominent fluorescence can be observed in the oral field. In the control cells without primary antibody (absolute control) there is no fluorescence at all, using usual (1%) laser intensity and this appears only at 30% (Fig. 1 A). In this case there is fluorescence in the vacuoles of the body, however there is only a very pale lightening in the cortical region. This means that the fluorescence demonstrated in the case of complete immunocytochemistry is specific, demonstrating the presence of the hormone.

One hour after treatment there is a stronger than control fluorescence in the chorion gonadotropin (Figs. 2 A, B), FSH (Figs 2 C, D), FSH+LH (Figs. 2 E, F) and TSH (Figs. 2 G, H) treated cells. The most intensive cortical, oral field and ciliary fluorescence can be observed in the HCG and TSH treated cells.

Twenty four hours after treatment the intensity of fluorescence is seems to be more intensive in each case, than it was 1 h after treatment (Fig. 3). The strongest reaction is present in the case of FSH+LH (Figs. 3 E, F) and TSH (Figs. 3 G-I) treatment. The fluorescence of cilia and kinetids is striking. The diffuse fluorescence present in the control cells and 1 h after treatment disappears in the LH and FSH treated cells.

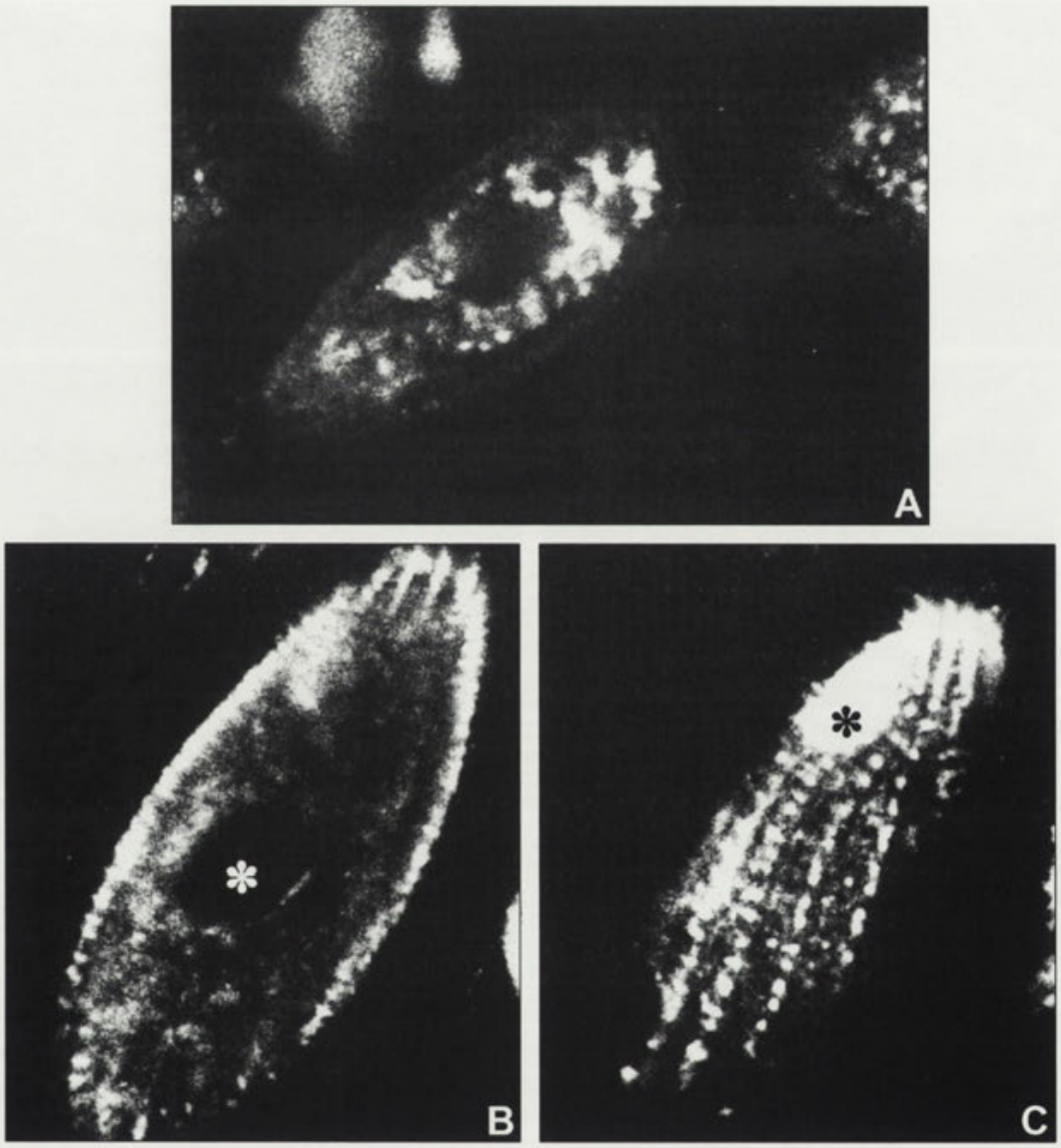


**Fig. 1.** Control preparations: **A** - absolute control (only secondary antibody was used). Intensity of laser-light for making the photo is 30-time of the laser light used in case of other photos. **B** - control (primary and secondary antibody), median plane; **C** - control, surface. White * - nucleus; black* - oral field

Four days after treatment the fluorescence of the cortical region, oral field and cilia are more intensive than in the control cells. The most intensive reaction is given again by the HCG and TSH treated (cells Figs. 4 E-H). The diffuse fluorescence present in the control cells and 1 h after treatment also can not be observed in the LH or FSH treated cells: the cell body is fluorescence-free.

## DISCUSSION

The results clearly show the presence of immunologically HCG-like materials in *Tetrahymena*. Considering the cross reactions of the antibody it seems to be difficult to determine the exact quality of the hormone. The cross reactions are understandable: the α-subunits of the pituitary glycoprotein hormones are identical, while the β-subunits have an about 80% similarity, except growth hormone, which has a 60% similarity to the other members of the family (Frieden 1976, Muyan *et al.* 1998).

The unicellular *Tetrahymena* contains many molecules, which are immunologically or functionally similar to mammalian hormones. In the late sixties Blum (1967) demonstrated an adrenergic system in *Tetrahymena*, later on the basis of our receptor experiments (Csaba

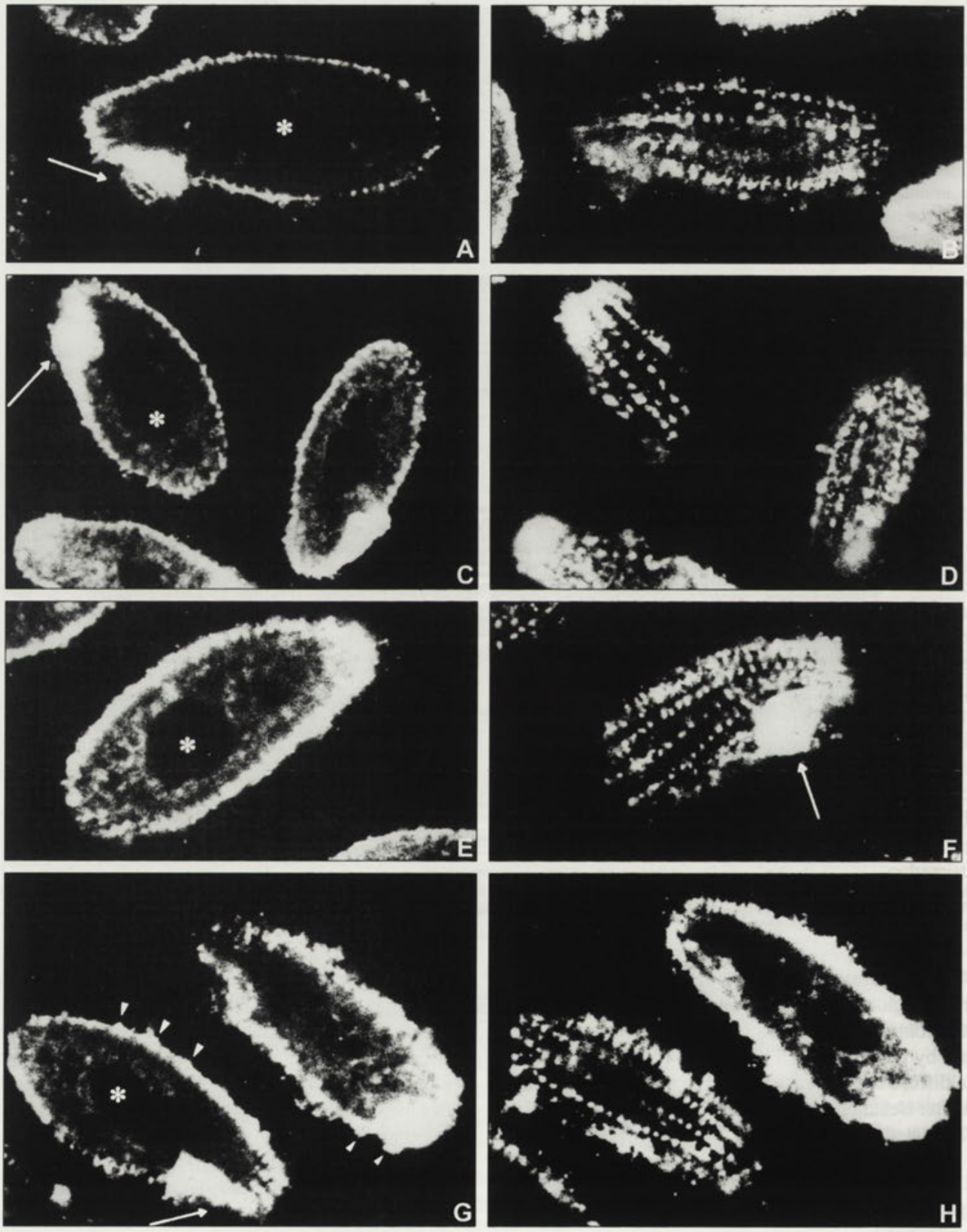


**Fig. 2.** At the end of the 1 h treatment with **A**, **B** - chorionic gonadotropin hormone; **C**, **D** - follicle stimulating hormone; **E**, **F** - luteinizing hormone and follicle stimulating hormone; **G**, **H** - thyrotropic hormone. Left side - median plane, right side - surface. Arrow - oral field; * - nucleus; arrowhead - aggregated cilia and kinetids

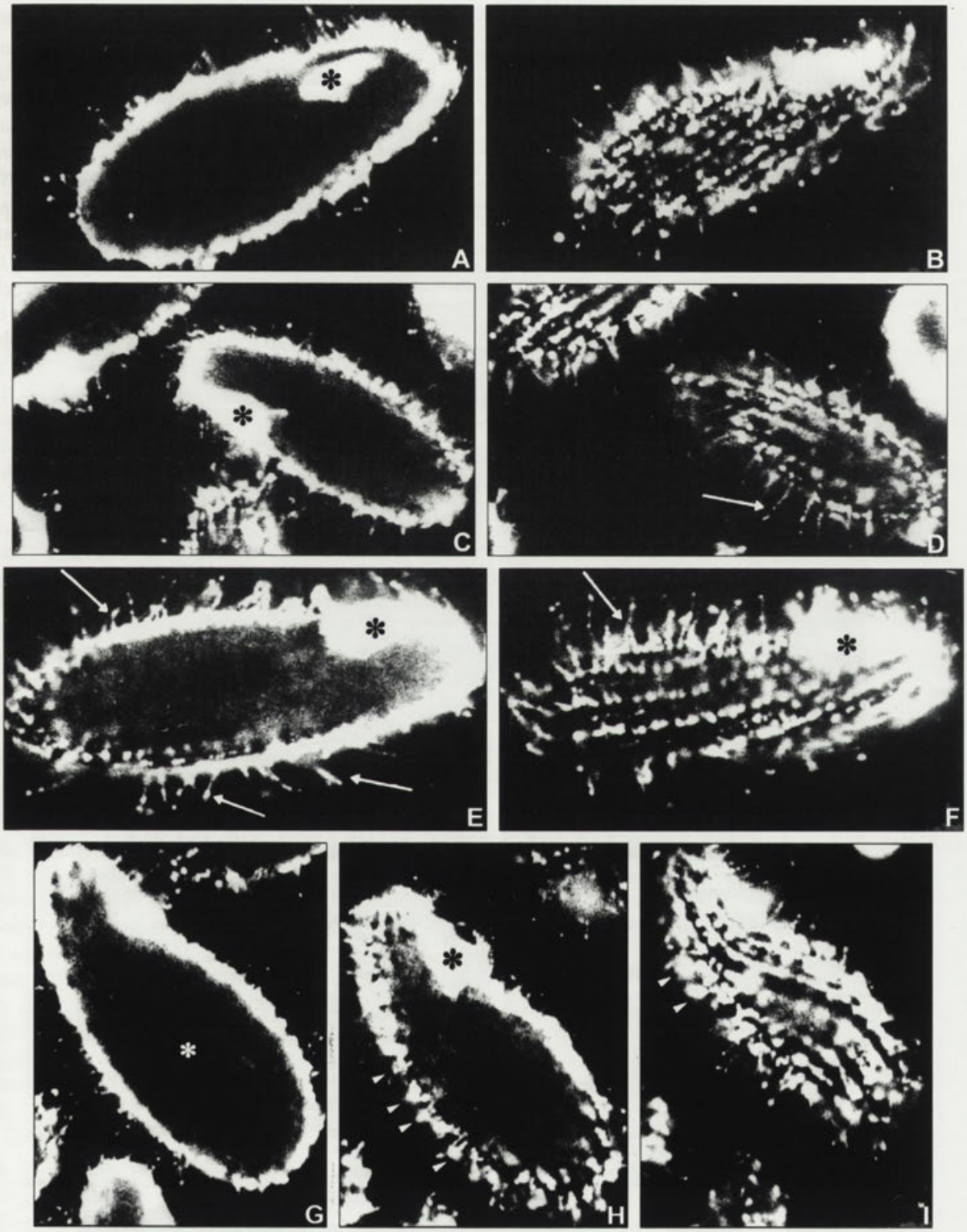


**Fig. 3.** One day after treatment with: **A, B** - chorionic gonadotropin hormone; **C, D** - follicle stimulating hormone; **E, F** - luteinizing hormone and follicle stimulating hormone; **G, H, I** - thyrotropic hormone. Left side - median plane, right side - surface (**H** is sectioned near to the surface). Black* - oral field; white* - nucleus; arrows - cilia; arrowheads - kinetids

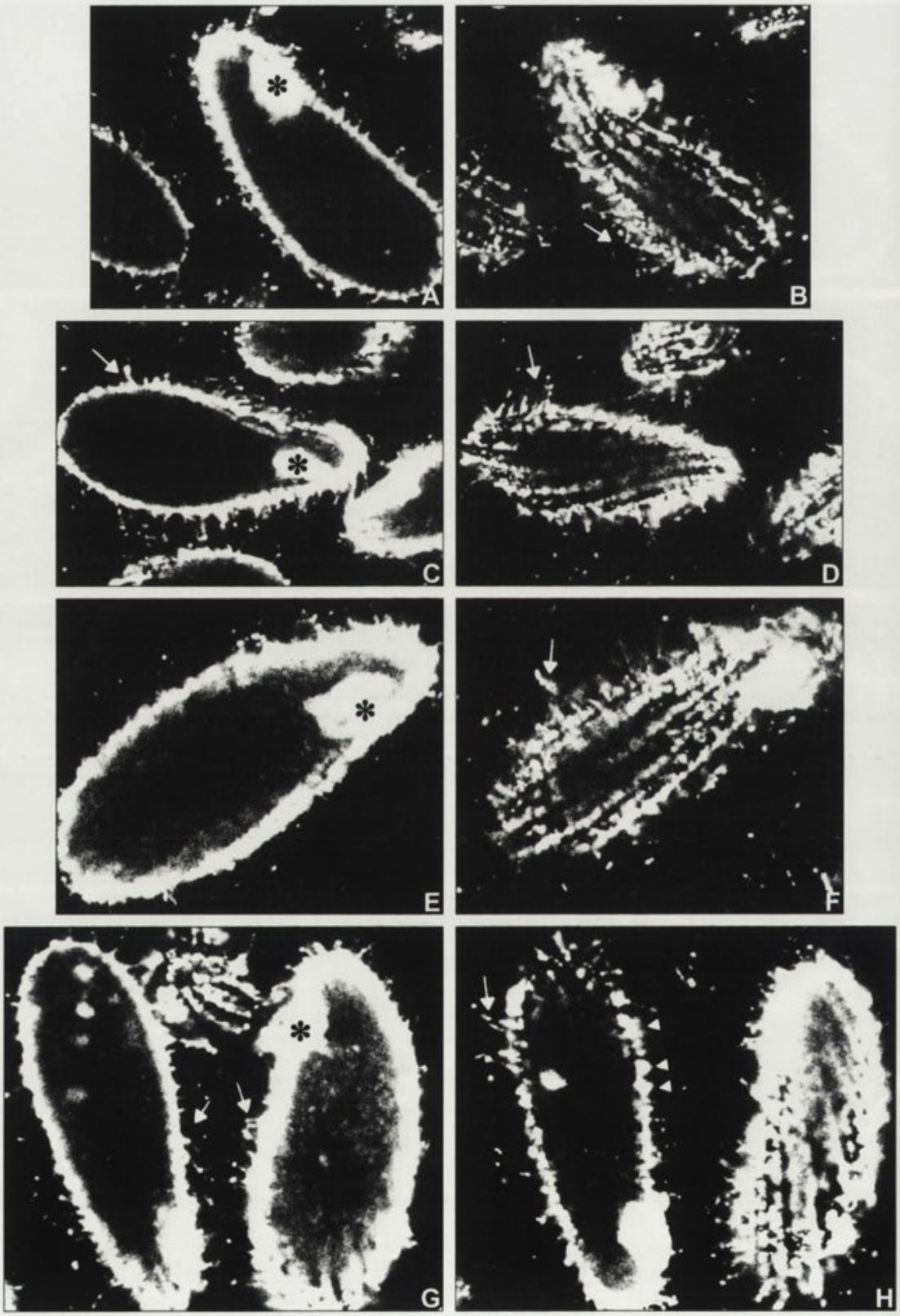


**Fig. 4.** Four days after treatment with **A**, **B** - chorionic gonadotropin hormone; **C**, **D** - follicle stimulating hormone; **E**, **F** - luteinizing hormone and follicle stimulating hormone; **G**, **H** - thyrotropic hormone. Left side - median plane; right side - surface (except **G**, **H**, where more cells can be seen); black* - oral field; arrow - cilium; arrowheads - kinetids

1980) Roth's group studied and found the presence of insulin-, somatostatin-, relaxin-, β-endorphin-, arginin vasotocin-, and calcitonin-like molecules (LeRoith *et al.* 1980, 1882, 1983; Roth *et al.* 1982), and we found histamine (Hegyesi *et al.* 1999), serotonin (Csaba and Kovács 1994) and steroid hormones (Csaba *et al.* 1985, 1998). Of the pituitary hormones, presence of ACTH was demonstrated (LeRoith *et al.* 1982) and as a pituitary hormone like molecule, human placental lactogen (Csaba and Nagy 1987). Of the hormones studied, only triiodothyronine and thyroxine was not found (Csaba and Nagy 1987). This means, that the presence of molecules demonstrable by HCG-antibody is not surprising.

It is not known what is the role of these hormone-like materials in *Tetrahymena*. Considering that receptors and signal transduction systems also can be found (Kuno *et al.* 1979; Csaba 1985, 1994; Köhidai *et al.* 1992), they could be tools for an autoregulation. As the sensitivity of receptors is high enough, especially after imprinting (Csaba *et al.* 1982 b), they could be also tools of intercellular communication (LeRoith *et al.* 1987, Nobili *et al.* 1987). However, they would be side-products of the protein synthesis of a unicellular organism which -being a ciliate- is at the top of the unicellular evolution (Hill 1972).

From our point of view it is more interesting the study of imprinting. The results show that 24 or 96 h after treatment there is a more intensive hormone fluorescence (more hormone) in the cells, than immediately after treatment. This makes likely that *Tetrahymena* produced and stored the hormone-like molecules, which supports earlier results on the prolonged enhancement of hormone production induced by the hormonal imprinting (Csaba *et al.* 1999). The enrichment of the hormone can be observed in the surface and median sections alike. In the case of the surface it can be supposed that it is a consequence of the receptorial imprinting: more receptors are present which bind larger amount of the secreted hormone (maybe this is shown by the stronger reaction of the cilia). However, the median sections clearly demonstrate the higher quantity of the hormone also inside the cell.

The localization of the HCG-like material is interesting. In the non-imprinted (untreated control) cells there is diffuse localization in the cell body and a dense localization in the cortex; and the same is observed immediately after treatment (1 h). This type of localization can be observed also after 24 and 96 h in the HCG or TSH treated cells. However, in the LH and FSH treated cells there is a complete rearrangement of the hormone localization: the cell body is cleared and the fluorescence of the cortical region strengthened. The reason of this phenomenon is not known. The strong reaction of the oral field can be explained by the presence of many cilia as well, as by the reuptake of the secreted hormone.

It is worth to mention that after 24 and 96 h the strongest reaction was observed in the LH+FSH and TSH treated groups, and after TSH treatment was the most intensive one. This could be understandable in the case of LH+FSH. However, considering that the cross-reaction of HCG-antibody to LH is weaker than to HCG and to LH is larger than to TSH, this makes likely that *Tetrahymena* can select between the uptake of the related hormones or in the execution of imprinting. Nevertheless, the method used is suitable first of all for demonstrating qualitative differences, so this statement must be administered cautiously.

The experiments clearly show that the unicellular *Tetrahymena* contains one or more types of molecules, which are immunologically similar to the glycoprotein hormones of the human pituitary gland. Treatment (hormonal imprinting) with these molecules incites the cells for producing the appropriate hormone as was done previously in the case of insulin (Csaba and Kovács 1995, 1999) and endorphin (Csaba and Kovács 1999). Considering the growth rate of *Tetrahymena*, at 96 h about the 30th generation is living. This means that the effect of hormone-inducing imprinting is transmitted to the progeny generations.

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# A Coccoid Bacterial Parasite of *Naegleria* sp. (Schizopyrenida: Vahlkampfiidae) Inhibits Cyst Formation of its Host but not Transformation to the Flagellate Stage

**Rolf MICHEL[1], Karl-Dieter MÜLLER[2], Bärbel HAURÖDER[1] and Lothar ZÖLLER[1]**

**[1]Central Institute of the Federal Armed Forces Medical Services, Koblenz; [2]Institut für Medizinische Mikrobiologie, Universität Essen, Essen, Germany**

**Summary.** A non-thermophilic *Naegleria* species isolated from an aquarium for ornamental fishes harboured coccoid Gram-negative bacteria of 1µm in diameter as endocytobionts multiplying by binary fission. These intracytoplasmic bacteria, called "KNic" inhibited cyst formation of their host, but not its transformation to the flagellate stage. Consequently the resulting flagellates contained these intracellular parasites as could be verified by electron microscopy. The endocytic bacteria could not be grown on eight different bacteriological media under various atmospheric conditions. The ribbon-like roughly hexagonal surface of the bacteria as shown by SEM is responsible for the spiny appearance as seen by TEM. The successful axenisation of the infected host amoebae made it possible to harvest pure bacterial suspensions for cocultivation assays with different free-living amoebae and *Dictyostelium discoideum*. As a result, an extremely wide host spectrum of the endocytobionts could be determined including other *Naegleria* spp., acanthamoebae, and members of the genera *Hartmannella, Vahlkampfia*, and *Balamuthia*. Successful infection of four additional *Naegleria* strains with the coccoid endocytobionts resulted also in impairment of the capability to form cysts but did not interfere with transformation to the flagellate stage. *Dictyostelium discoideum* as a member of the Acrasiales was also highly susceptible to infection with the Gram-negative bacteria inducing a strong disturbance of their normal aggregation pattern.

**Key words:** amoeboflagellate, cyst formation, endocytobionts, Gram-negative cocci, host range, *Naegleria* sp., ultrastructure.

## INTRODUCTION

The multiplication of bacteria within free-living amoebae (FLA) resulting in lysis or rupture of the host cell has been known since the observations of Nägler (1910). In recent years, however, interest in the interaction between bacteria and free-living amoebae has increased as a result of the first findings of Rowbotham (1980), who observed the infection of acanthamoebae by pathogenic *Legionella pneumophila* and later "legionella-like amoebal pathogens" (Llaps) (Rowbotham 1993). These relations were subject to investigations of many authors (Rowbotham 1987, Adeleke *et al.* 1996, Birtles *et al.*1996, Michel *et al.* 1998). Various *Legionella* spp. usually multiply within vacuoles

Address for correspondence: Rolf Michel, Central Institute of the Federal Armed Forces Medical Services, P.O. Box. 7340, D - 56065 Koblenz, Germany; Fax: +49 261/ 896 1306; E-mail: rolf_michel@hotmail.com

(phagosomes) of acanthamoebae, naegleriae, and hartmannellae - whereas Llaps multiply within the host cytoplasm. In addition, *Listeria* spp. (Ly and Müller 1990) and certain *Pseudomonas* spp. (Michel and Hauröder-Philippczyk 1992, Michel and Hauröder1997) multiply intracellularly mainly within acanthamoebae. In contrast to these Gram negative rod-shaped bacteria, non-cultivable *Chlamydia*-like bacteria were observed within acanthamoebae isolated from the human nasal mucosa (Michel *et al.* 1992, 1994) that turned out to belong to the novel genus *Parachlamydia* as shown by DNA-sequencing (Amann *et al.* 1997). In a new taxonomic approach this genus was attributed to the newly created family "Parachlamydiaceae" (Everett *et al.* 1999) within the order Chlamydiales. Several strains of *Chlamydia*-like bacterial parasites within acanthamoebae have also been described by Birtles *et al.* (1997) and Fritsche *et al.* (1998).

Very few reports exist on the occurrence of endocytobionts in other genera except *Acanthamoeba*. *Ehrlichia*-like parasites from *Saccamoeba* (Michel *et al.* 1995), microsporidia-like endocytobionts from *Vannellae* (Hoffmann *et al.*1997) and yeast- like organisms parasitizing *Thecamoeba similis* (Michel 1997) have only recently been described.

A novel coccoid endocytobiont with *Naegleria* as host amoeba is object of the following description dealing with its isolation, morphology and transmissibility to other FLA and behaviour during the flagellation process of its host cell.

## MATERIALS AND METHODS

### Sources of amoeba strains

Most strains of FLA tested as possible hosts for the novel endocytobiont strain, called KNic, and were isolated in our laboratory. The corresponding source is indicated in Table 1.

We are indebted to Johan De Jonckheere for providing us with *Naegleria lovaniensis* strain Aq/9/1/45D, to Klaus Janitschke for the *Balamuthia* strain "CDC:VO39" and to Iris Weishaar for providing the *Dictyostelium* strain "Berg 25" - that was originally isolated from the human nasal mucosa.

### Isolation of infected naegleriae

100ml water from a freshwater aquarium for ornamental fishes was filtered through a Sartorius-filter with 0.45 µm pore size. The filter was placed upside down onto the surface of an NN-agar plate (Page 1976) streaked with one drop of a 24h culture of Enterobacter *cloacae* as food source. For the attempted reisolation several mud samples were placed on the agar surface.

Within a period of one week an unusual colony of naegleroid amoebae unable to form cysts was transferred as a pure culture to a fresh agar plate. After the observation of coccoid bacteria within the cytoplasm of the trophozoites by means of phase contrast microscopy several attempts to get this amoebic host strain free from accompanying bacteria in SCGYE-medium (De Jonckheere 1977) showed that it was not able to grow axenically. Consequently, filter-purified endocytobionts (1.2 µm pore size) from host cells submitted to freeze thawing were cocultivated together with *N. lovaniensis* - a strain known to grow readily under axenic conditions. Since this experimental host could easily be infected on NN-agar plates the trophozoites were subsequently retransferred to liquid SCGYE - medium supplemented with penicillin and streptomycin. As the new hosts retained their endoparasites, they were harvested, centrifuged at 1800 rpm, and submitted to electron microscopy.

The flagellate transformation test was performed with trophozoites from 3-5 day-old cultures of strain "NBeck" on NN-agar. They were suspended with amoebic saline according to Page and observed for 2 -3 hours. When flagellates were formed they were fixed for electron microscopy 1 : 2 with 6% glutaraldehyde in cacodylate buffer. After one hour they were centrifuged at 2500 rpm and resuspended in 0.1 M cacodylate buffer, then treated the same way as the trophozoites.

### Cocultivation assay

Axenic cultures of *N. lovaniensis* harbouring KNic were harvested after 5 days when nearly all host amoebae had been killed so that the medium contained numerous free cocci that were purified by filtration through a membrane filter with a 5 µm pore size (Sartorius AG, Göttingen, Germany). 50 µl of the suspension were added to log-phase cultures of FLA and monitored for at least 8 days.

In order to test the susceptibility of the original host for its own endoparasitic bacteria the infected original isolate was cured from its parasites by treatment with a mixture of penicillin and streptomycin (0.1 g/ml each) on NN-agar.

A successful infection of experimental host cells could easily be identified directly by means of phase contrast microscopy and indirectly by recognition of damaged host cells as a result of massive infection.

### Electron microscopy

**TEM-preparation.** Trophozoites for electron microscopy were harvested from 3 day-old axenic cultures, fixed in cacodylate buffered 3% glutaraldehyde, postfixed in 1 % $OsO_4$, and stained with uranyl acetate and lead citrate. Sections were examined in a LEO 910 transmission electron microscope (Leo, Oberkochen).

**SEM-preparation.** Aliquots of a five day-old culture containing numerous free cocci were freeze-thawed, passed through a membrane filter (Sartorius, 5µm) and fixed in cacodylate buffered glutaraldehyde (3%, 1h). After washing in 0.1M cacodylate buffer, endocytobionts were collected on a polycarbonate filter (Nucleopore, 0.2 µm). Samples were then dehydrated in a graded series of ethanol and critical point dried using $CO_2$. Subsequently the samples were sputtered with gold and photographed with a LEO 1350 scanning electron microscope (Leo, Oberkochen).

**Table 1.** Coccoid endocytobionts (KNic) of *Naegleria* sp., evaluation of the host range

| Host-species | Strain | Source | Intracytoplasmatic multiplication |
|---|---|---|---|
| *Naegleria* sp. | Nic (orig. Stamm) | aquarium | + |
| " | N30/40 | sewage | + |
| " | NiPi3 | surface water | + |
| " | RJTM | Philippines patient | + |
| *N. lovaniensis* | Aq/9/1/45D | aquarium | + |
| *Willaertia magna* | NI4C11 | India pond | + |
| " | PAOBP40 | Spain brook | + |
| *Hartmannella vermiformis* | Hspio | Dental unit | + |
| " | Os 101 | hospital tap water | + |
| *Acanthamoeba castellanii* (II) | C3 | potable water reservoir | + |
| *A. quina-lugdunensis* (II) | 312-1 | nasal mucosa | + |
| *A. astronyxis* (I) | Am23 | Physiotherapeutic unit | - |
| *A. comandoni* (I) | Pb40 | greenhouse | - |
| *A. lenticulata* (III) | 45 | nasal mucosa | - |
| *A. lenticulata* (III) | 89a | nasal mucosa | + |
| *Vahlkampfia ustiana* | A1PW | Egypt (Nile river) | - |
| *V. ovis* | Rhodos | puddle | + |
| *Flamella aegyptia* sp. n. | A1, 3 | Egypt (Nile river) | - |
| *Comandonia operculata* | WBT | raw water reservoir | - |
| *Phreatamoeba*-like amoeba | KB | Danmark brook | - |
| *Balamuthia mandrillaris* | CDC:VO39 | *Papio sphinx*: brain | + |
| *Dictyostelium discoideum* | Berg 25 | nasal mucosa | + |

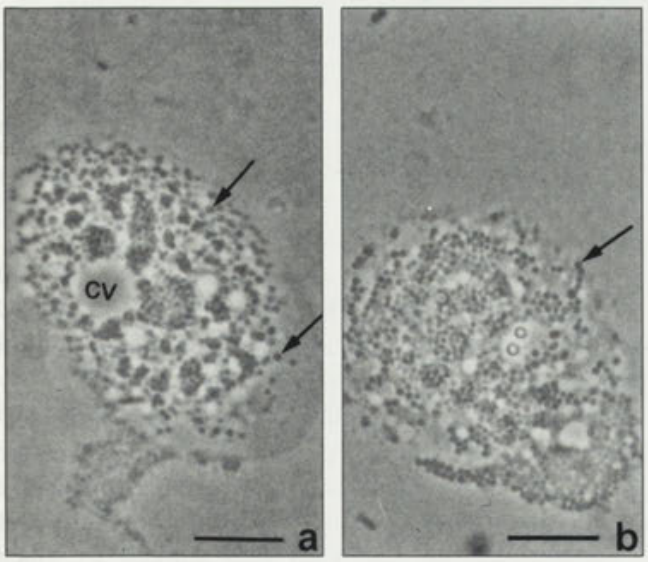


**Fig. 1.** Light microscopic appearance of two heavily infected trophozoites of *Naegleria* sp. with coccoid bacterial parasites (KNic) (arrows). The left trophozoite (**1a**) is still alive, as documented by a still operating contractile vacuole (cv). Scale bars - 10µm, 1200x

## Cultivation of KNic on acellular media

In an attempt to cultivate KNic on synthetic media, suspensions of filtered endocytobionts were inoculated onto various standard agar and broth media and cultivated for two weeks under aerobic and anaerobic conditions at various temperatures.

In detail, cultivation conditions were as follows: Columbia agar with 5% sheep blood, room temperature and 37°C, aerobic atmosphere with 10% $CO_2$; chocolate agar, room temperature and 37°C, aerobic atmosphere with and without 10% $CO_2$; Schaedler agar, room temperature and 37°, aerobic atmosphere with and without 10% $CO_2$ and anaerobic atmosphere; brain heart infusion agar (BHI), room temperature and 37°C, aerobic atmosphere; BCYE agar at room temperature and 37°C, aerobic atmosphere; glucose peptone broth with one drop of fetal calf serum at room temperature and 37°C, aerobic atmosphere;

BSK (Barbour-Stoenner-Kelly) medium (broth) at room temperature and 37°C; aerobic atmosphere; LB medium (Luria broth) at room temperature and 37°C, aerobic atmosphere.

## RESULTS

A non-thermophilic naegleroid amoeba isolated from an aquarium for ornamental fishes attracted our attention by its inability to form cysts. Since for that reason morphological features of the cyst were not available, the trophozoites were submitted to a flagellate - transformation test at room temperature in order to decide if these amoebae exhibiting naegleroid movement were members of the genera *Vahlkampfia* or *Naegleria*. Within two hours a considerable number of trophozoites

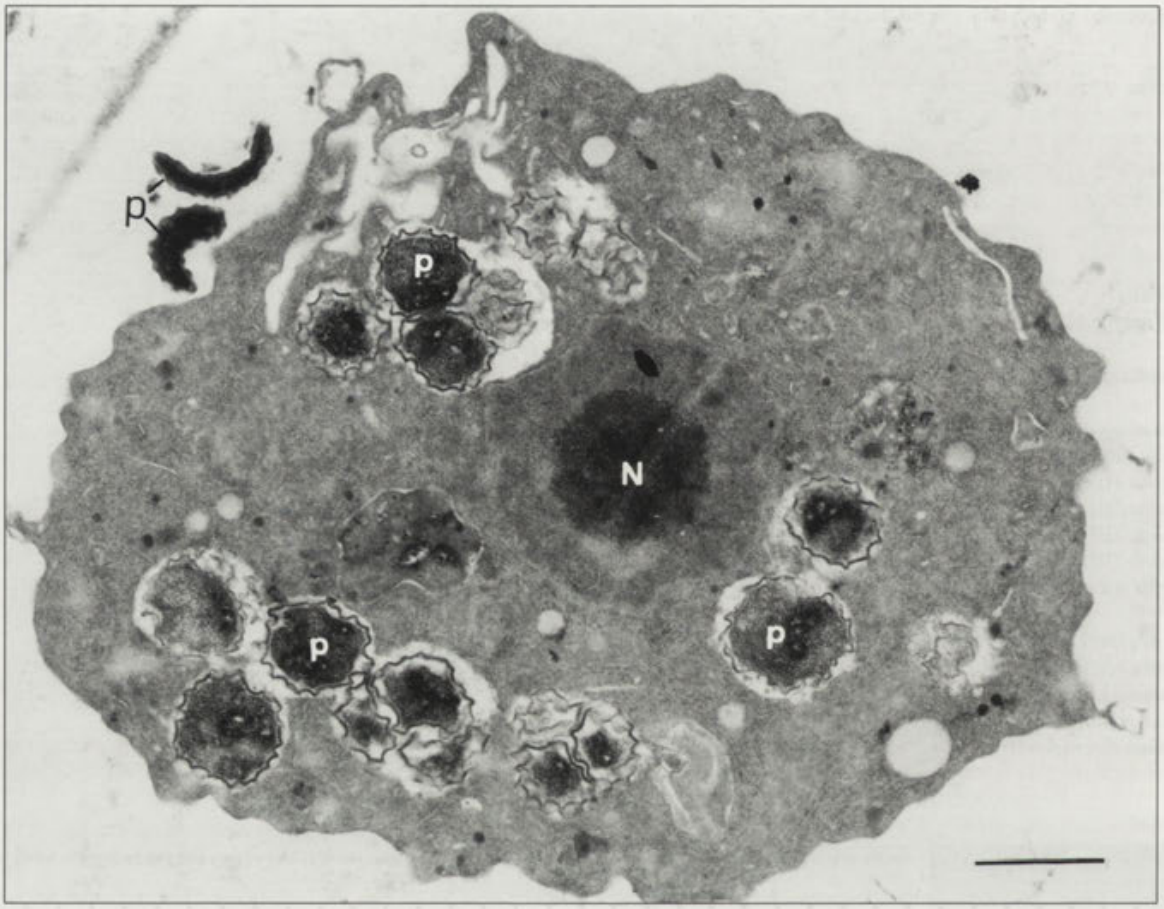


**Fig. 2.** *Naegleria lovaniensis* after transfer of endocytobionts from original host strain showing 10 - 12 endocytobionts (P), distributed freely within the cytoplasm surrounded by an electron translucent area. The Gram-negative roughly coccoid stages exhibit a spiny appearance. The host amoeba is not damaged at this stage of infection. Two crescent-shaped parasites (P) are located outside the amoeba. N - nucleus of the host amoeba. Scale bar - 1µm, 16800x

had transformed to the flagellate stage proving that this strain belonged to the genus *Naegleria*. But why did they not form cysts? This puzzling question was solved first by observation of trophozoites by phase contrast microscopy (objective 100x) (Fig. 1). Most of the trophozoites contained a great number of coccoid organisms distributed singly or in groups within the cytoplasm, thus being clearly distinguishable from mitochondria. Heavily infected cells exhibited aggregates of those cocci resulting in rupture or lysis of the host cell (Fig. 1). Moderately infected amoebae were found to continuously secrete cocci into the environment, leaving a trace of free cocci behind them while moving into one direction, as has recently been shown provisionally for different kinds of endocytobionts (Michel *et al.* 1998). These intracellular organisms were bigger than cocci of the new genus *Parachlamydia* parasitizing acanthamoebae. Measurements performed by means of electron micrographs showed a diameter of 1µm on an average in contrast to 0.5 µm of *Parachlamydia.*

The investigation of thin sections by means of electron microscopy revealed the precise shape and nature of these cocci. They were Gram-negative and had a spiny appearance. Figure 2 shows a section of *N. lovaniensis*, the secondary host amoeba to which these endocytobionts had been transferred from their original host strain "Nic". This measure was necessary because the original host was not able to grow axenically in SCGYE-medium. Within the plane of this section (Fig. 2) 10-12 spiny cocci can be seen single or in small groups distributed freely within the cytoplasm, some of which are surrounded by an electron translucent area. No continuous vacuolar membranes could be observed. The host amoeba did not show any signs of damage at

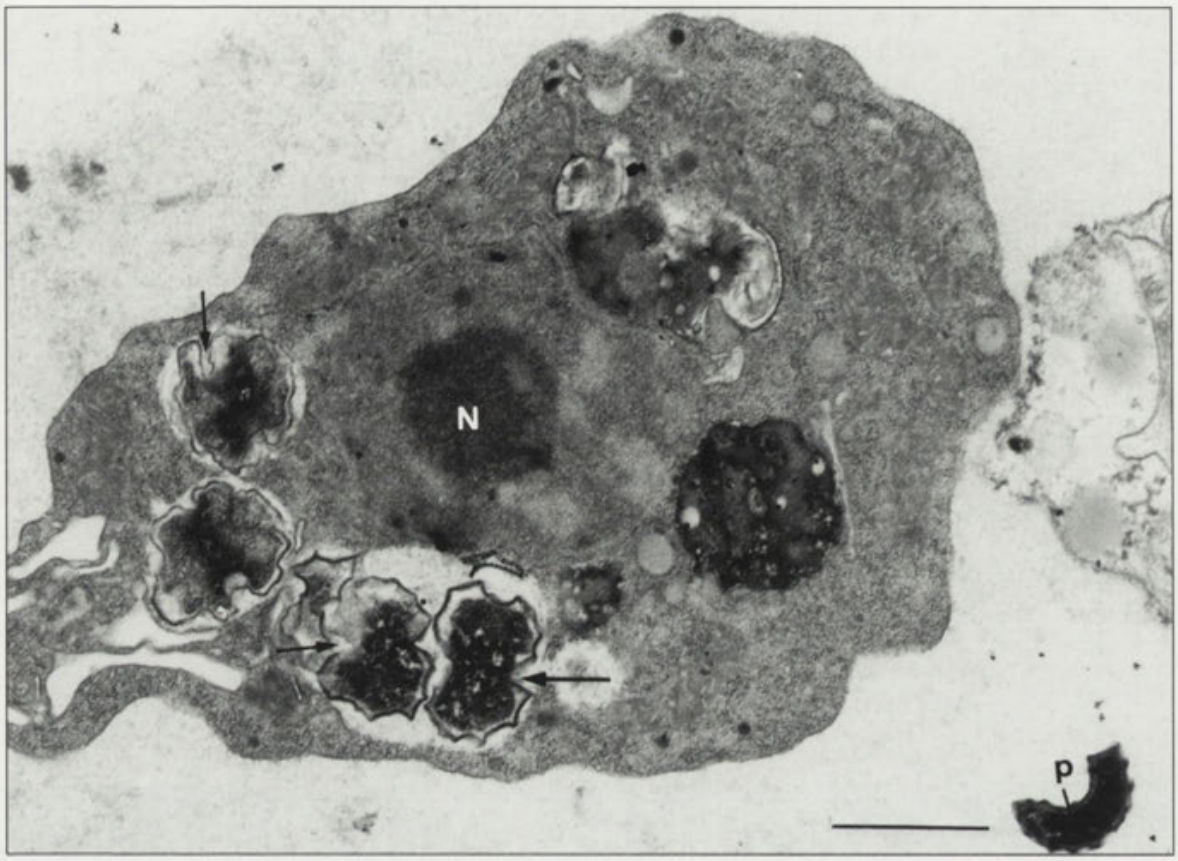


**Fig. 3.** *Naegleria lovaniensis* harbouring 4 endocytobionts showing binary fission (arrows) indicated by the constriction during fission. Eventually two parasites on the right side succumb to digestion by their host. Scale bar - 1μm, 21400x

this stage of infection. Two crescent-shaped parasites (P) are located outside the amoeba, the unusual shape possibly being an artifact. In other sections of the same host cells (Fig. 3) stages of binary fission without septum formation can be observed. It seems that in rare cases some endocytobionts succumb to digestion by their host amoeba (Fig. 3).

At an advanced stage of infection the entire cytoplasm of the host cells appeared crowded by numerous cocci pushing the nucleus (Fig. 4) and contractile vacuoles (not shown) into the marginal cytoplasm - in the same way as it was shown earlier with *Parachlamydia* endocytobionts of acanthamoebae.

**Attempts to cultivate KNic on acellular media**

A broad range of different agar and broth media were inoculated with KNic in order to evaluate their capability to grow on acellular media.

None of the cultures showed signs of bacterial growth at the end of the incubation period, independently of incubation conditions. These findings suggest that KNic are noncultivable on synthetic acellular media. The not impaired ability of the original host cell to transform to its flagellate stage despite massive infection led to two different hypotheses explaining this behaviour; the most simple explanation would be that only hitherto uninfected trophozoites of the population were able to transform. The more unlikely assumption would be that even infected trophozoites were capable to transform into flagellates. In order to elucidate this, we transferred the parasitic organisms to "NBeck" a *Naegleria* strain known to transform into flagellates at a high percentage (80%). This infected strain was submitted to the flagellate transformation test at room temperature. As a result the transformation was not adversely affected in spite of heavily infected trophozoites of this strain.

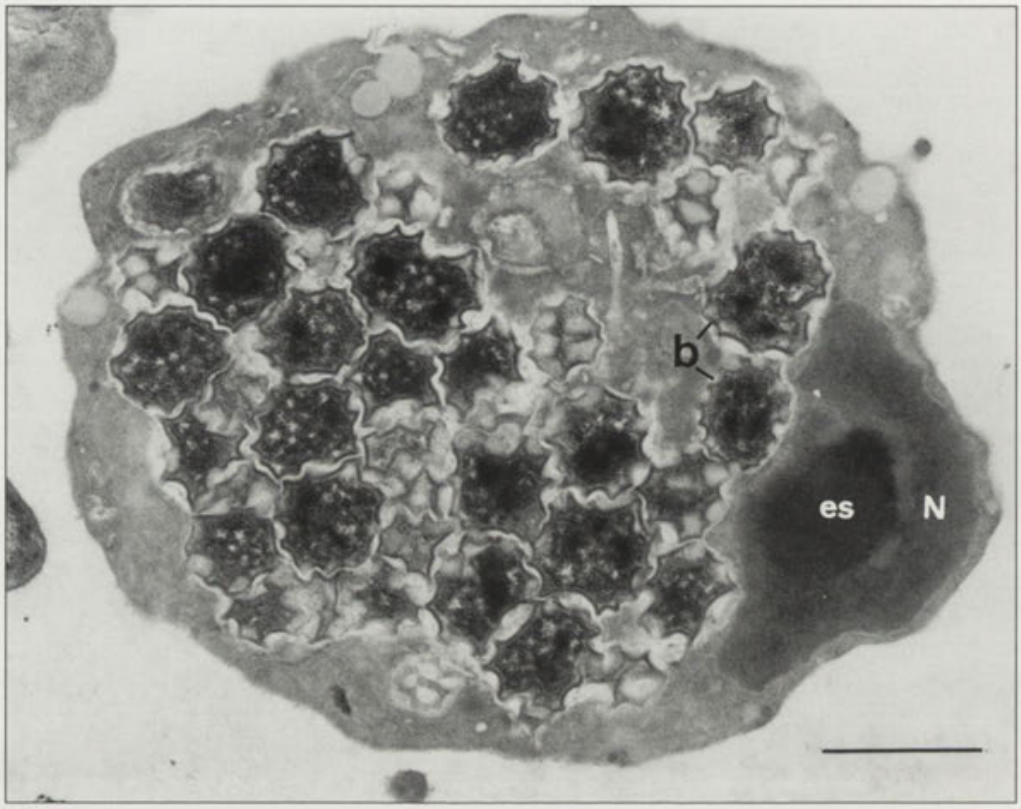


**Fig. 4.** *Naegleria* host amoeba at the final stage of infection crowded by numerous coccoid stages with spiny appearance (b). The nucleus (N) with its endosome (es) is pushed to the marginal cytoplasm of the amoeba. The amoeba is ready to burst at the next moment. Scale bar - 1μm, 21 400x

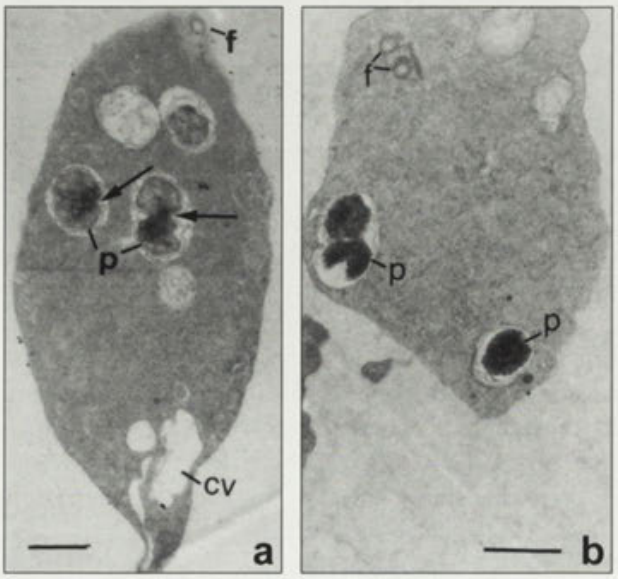


**Fig. 5.** Two flagellate stages harbouring intact endocytobionts (P): **a** - with two bacterial stages with binary fission (arrows). cv - contractile vacuole, f - cross-sectioned flagellum. Scale bar - 1μm, 8 000x; **b** - flagellate with three parasites showing cross-sectioned flagellae. Scale bar - 1μm, 10 200x

The flagellates were fixed and submitted to electron microscopy as described above. Nearly all sections (Fig. 5) contained flagellates harbouring one or more endocytobionts with the same spiny appearance as seen before with some of them undergoing binary fission. The findings proved that multiplication of the bacteria continues even within the flagellate stages. The latter could be distinguished with ease from trophozoites by having flagellae and a homogeneous cytoplasm. Considering the spiny shape of the intracellular parasites, a suspension of filter-purified cocci was submitted to scanning electron microscopy (Fig. 6). Both types of cocci - those with a smooth surface (s) and those with a rough envelope (arrows) could be observed within the same area of the filter surface - the latter type corresponding to intracellular organisms as seen before by means of TEM. A considerable number appeared indented (i), possibly an artifact caused by a hypertonic medium. At higher magnification the rough surface structure exhibited a pattern resembling arrays of honeycombs. Intact parasites therefore bear a strong resemblance to golf balls.

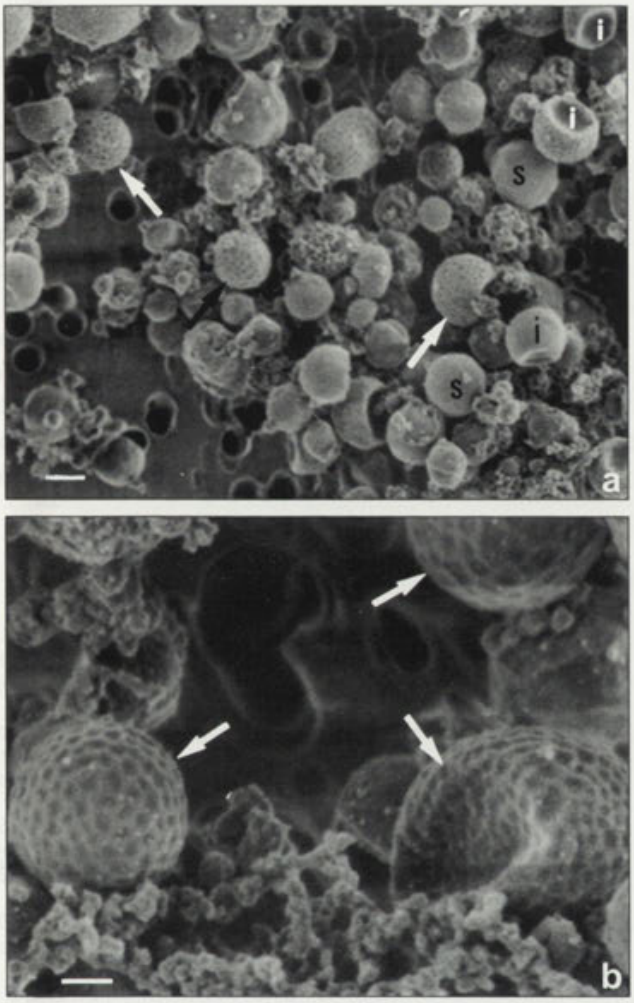


**Fig. 6.** Filter-purified endocytobionts "KNic" demonstrated by SEM. **a** - at this magnification cocci with rough (arrows) and smooth surface (s) can be observed, some of them being indented (i). Scale bar - 0.5µm, 11 000x; **b** - at higher magnification rough cells (arrows) exhibit a sculptured surface covered with honeycomb-like structures. Scale bar - 0.2µm, 35 000x

## Evaluation of the host spectrum

In order to answer the question of whether or not this endocytobiont, which was originally isolated from a *Naegleria* species would also be able to multiply within other *Naegleria* species or even in FLA of other genera, a filter-purified suspension from a heavily infected *N. lovaniensis* culture was cocultivated with 21 different strains of FLA and one strain of the social amoeba *Dictyostelium* (Table 1). In addition the parasite-free host strain obtained by treatment with antibiotics was cocultivated, too, resulting in a successful intracytoplasmic multiplication as had been seen in the original isolate. Four additional *Naegleria* strains from different sources were also cocultivated including two thermophilic strains. They were all susceptible to infection with the coccoid organisms. Trophozoites infected in this way also lost their capability to form cysts - but not the ability to transform to the flagellate stage!

It was reasonable to at first submit other genera of the same order Schizopyrenida, such as *Willaertia* and *Vahlkampfia*, to this cocultivation assay. Both *Willaertia* strains were as susceptible as *Naegleria*. But only one of two *Vahlkampfia* strains used for this purpose could be infected. Furthermore both *Hartmannella* strains - only distantly related - were also susceptible. Of special interest is the diverse reaction of 6 *Acanthamoeba* strains, as members of this unrelated genus are known as suitable hosts for a great number of reported endocytobionts. Two strains of group II- and only one out-of two group III- acanthamoebae could be easily infected, whereas two members of group I were resistant. Of four further genera rarely found in the environment only the new facultatively pathogenic species *Balamuthia mandrillaris*, first isolated from *Papio sphinx* was highly susceptible to these endocytobionts from *Naegleria*. Within 3-5 days nearly all trophozoites succumbed to the infection. After repeated transfer of single surviving amoebae to fresh medium they became more and more resistant to infection in the course of several transfers. Although still infected they excreted a great number of their parasites and regained their capability to divide. An intriguing result was the observation that also the amoebic stage of the slime mold *Dictyostelium discoideum* ingested the infectious stages of "KNic". The bacteria resisted digestion by the trophozoites, leading to a spread of the infection to the entire population of the solitary amoebae even before they began to aggregate. The parasitic organisms interfered distinctly with the aggregation behaviour leading to considerable disturbance of the subsequent development of the fruiting body (Michel, in preparation).

## Repeated demonstration of infected host cells

Finally we wondered whether the first isolation of the endocytobiont from the aquarium for ornamental fishes was a single event or whether it could be reproduced, which would indicate permanent infection of amoebae living within this aquarium. For this purpose, one year after the first isolation mud and plant material of it was transferred to NN-agar plates according to Page. Within a period of 8 days, populations of naegleriae, obviously not capable to form cysts, were isolated and exhibited intracellular cocci as seen before. This strain called "Nic 2a" was submitted to electron microscopy with the same

result in morphology (not shown) as seen before. Our findings proved that these special endocytobionts survived and multiplied continuously in this aquatic habitat for at least one year.

## DISCUSSION

The real nature of this Gram-negative coccus with the spiny appearance of its cell wall remains obscure, as all attempts failed to cultivate it on various media normally used to grow Gram-negative cocci like *Neisseria* species. These cocci have no similarity with the previously described *Chlamydia*-like genus *Parachlamydia* (Aman *et al.* 1997) or with other *Chlamydia*-like strains (Fritsche *et al.* 1998), because they do not have two different developmental stages i.e. "reticulate bodies" and "elementary bodies". Further identification trials must therefore concentrate on DNA-sequencing of phylogenetically relevant genes.

The results of the cocultivation assays exhibit a poor selectivity of the strain "KNic" for certain amoebic species. This is in contrast to the more pronounced host specificities of most of the other endocytobionts investigated: *Parachlamydia* strains "Bn9" and "Berg17" (Michel *et al.* 1994) e.g. can only multiply within acanthamoebae or possibly within *Comandonia*, the strain "$A_1$Hsp" was found to be confined to amoebae of the genus *Hartmannella* as host strains. The newly described intracytoplasmic rod "*Odyssella*" is also confined to *Acanthamoeba* strains (Birtles *et al.* 2000). However *Legionella pneumophila* - one of the first described endocytobionts - is known to grow in acanthamoebae, naegleriae and hartmannellae (Rowbotham 1980). Surprisingly distantly related or even unrelated genera as *Balamuthia* or *Dictyostelium* were very susceptible with the result of a considerable alteration of the aggregation pattern of the latter species. As demonstrated recently, *Dictyostelium* was the only - distantly related - species that was also susceptible to infection with the *Chlamydia*-like endocytobiont "$A_1$Hsp" (Horn *et al.* 1999). However, this intracellular parasite did not interfere with the normal aggregation behaviour of the social amoebae. But the adaptation of the leptomyxid amoeba *Balamuthia* to its endocytobiont "KNic" within a short period of time is also a remarkable phenomenon. The trophozoites regain normal cell division capacity by continuously excreting cocci, with the result that the supernatant liquid medium in the tissue culture flasks contains masses of excreted parasites. On the other hand, trophozoites obviously unaffected for a long period of time multiply at the bottom of the flask.

The results of cocultivation assays suggest a very wide host range and a weak selectivity of this obligatory intracellular bacterial parasite.

It is therefore most likely that these hitherto unknown cocci may be found within acanthamoebae or other genera in the near future.

Nothing is known about the question of whether or not these endocytobionts are of medical importance. To clarify this point would require appropriate trials to infect tissue cultures or experimental animals. Potential pathogenicity may be suspected by the low selectivity of KNic among a range of numerous different cells.

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# Studies on Six *Euplotes* spp. (Ciliophora: Hypotrichida) Using RAPD Fingerprinting, Including a Comparison with Morphometric Analyses

**Zigui CHEN[1], Weibo SONG[1] and Alan WARREN[2]**

**[1]Aquaculture Research Laboratory, College of Fisheries, Ocean University of Qingdao, Qingdao, P. R. China; [2]Department of Zoology, Natural History Museum, London, United Kingdom**

**Summary.** Species separation among six morphologically similar *Euplotes* spp. was investigated using random amplified polymorphic DNA fingerprinting (RAPD fingerprinting). Distinctly different banding patterns were obtained for each taxon (the band sharing index (D) among the six was 0.36-0.59), which indicates that all those concerned are well-defined species. Phylogenetic relationships among the six species were also analyzed using both RAPD fingerprinting and morphological analysis. Based on the molecular data, the ciliates were split into two clusters: *E. vannus-minuta-woodruffi-charon*, and *E. eurystomus-octocarinatus*. By contrast, a phenetic dendogram for these six species derived from morphometric characters indicated that they are divided into two rather different clusters, namely *E. vannus-minuta-charon* and *E. eurystomus-octocarinatus-woodruffi*. The most significant difference between the two analyses is that *E. woodruffi* and *E. charon* belong to two different clusters according to the dendogram based on morphology, but are closely related in the dendogram based on RAPD fingerprinting. The present investigation shows that RAPD fingerprinting is a useful method for species separation, but it seems to be of limited use for constructing phylogenetic relationships among the six species investigated. The results of the morphometric analysis support the view that the pattern of the frontoventral cirri, type of silverline system, arrangement of dorsal kineties and the structure of adoral zone of membranelles are important characters for determining phylogenetic relationships among species of *Euplotes*, whereas body shape and size are less significant.

**Key words:** *Euplotes*, morphometry, phylogenetic relationships, RAPD fingerprinting, species separation.

## INTRODUCTION

Reconstruction of phylogeny and species identification remain two of the main tasks for systematists working on ciliated protozoa. Conventional research techniques, such as morphological investigations based on light and electron microscopic analyses, behavioural studies, and mating tests with living strains *etc.*, have been the bedrock on which ciliate systematics is based. These techniques, however, have their limitations as evidenced by the numerous examples of taxonomic confusion cited in the literature (Tuffrau 1960, Curds 1975, Fleury *et al.* 1992, Prescott 1994, Kołaczyk and Wiąckowski 1997, Song and Bradbury 1997).

The introduction of biochemical and molecular techniques, such as enzyme electrophoresis, PCR/RFLP, DNA diversity, and small subunit ribosomal RNA

Address for correspondence: Weibo Song, College of Fisheries, Ocean University of Qingdao, 266003 Qingdao, P.R. China; Fax: +86 532 203 2283; E-mail: wsong@ouqd.edu.cn

(SSUrRNA) sequence comparisons, have helped to resolve some of the systematic problems within the phylum Ciliophora, particularly with respect to species separation and the reconstruction of phylogenetic relationships (Schlegel *et al.* 1988, 1991; Orias *et al.* 1991; Jerome and Lynn 1996; Kusch and Heckmann 1996; Lynn *et al.* 1999). Random amplified polymorphic DNA (RAPD) fingerprinting, which detects polymorphic fragments of DNA throughout the genome by the polymerase chain reaction, offers new possibilities for analyzing genetic diversity at genus and species levels and is particularly useful in this respect because previous sequence information is not necessary (Weish and McClellane 1990, Williams *et al.* 1990).

Traditionally, species separation and phylogenetic reconstruction within the genus *Euplotes* have mainly relied on morphological and morphometric studies (Tuffrau 1960, Carter 1972, Curds 1975, Dragesco and Dragesco-Kernéis 1986, Song and Bradbury 1997, Song and Wilbert 1997). However, it has long been known that some characters of presumed taxonomic value are quite variable, even among the descendents of a single individual (Gates 1978, Schlegel *et al.* 1988). Furthermore, many features overlap among species and this often yields great confusion for species separation. RAPD fingerprinting can provide useful guidance for species separation, and possibly for indicating interrelationships, among morphologically similar and dissimilar taxa within the genus *Euplotes* (Kusch and Heckmannn 1996).

The aims of the current work were; (i) to determine whether RAPD fingerprinting can be used for a clear separation of some closely related species of *Euplotes*, and; (ii) to compare RAPD fingerprinting with morphometric analyses as methods for examining the phylogenetic relationships among these species.

## MATERIALS AND METHODS

**Ciliate strains and culture.** Of the six *Euplotes* species used in the present study, five were isolated from environmental samples collected in or around Qingdao, P. R. China. The sources of the isolates were as follows: *E. woodruffi* and *E. eurystomus*, both from a small freshwater pond in the Zhongshan Park, Qingdao; *E. charon*, from a marine shellfish-farm pond in Xunshan, Qingdao; *E. minuta*, from a marine prawn-farm pond in Hongdao, near Qingdao; *E. vannus*, from the coastal waters off Taipingjiao, Qingdao. Clonal cultures of each were established in the laboratory. Cultures were uniprotistan, the only other organisms present being the bacterial food sources. In the case of *E. octocarinatus*, genomic DNA was kindly provided by Dr. Liang Aihua (University of Shanxi, Taiyuan, China) and it was on this material that RAPD fingerprinting was carried out.

**Table 1.** Oligonucleotide sequences of primers for RAPD reaction

| Primer | 5' to 3' sequence |
|---|---|
| E1 (22bp) | ATG TAA GCT CCT GGG GAT TCA C |
| No.832 (17bp) | GGA AGA ATA CAG CAG CA |
| S101 (10bp) | GGT CGG AGA A |

**DNA extraction and PCR reaction.** Amplifications were performed using either 30 ng of genomic DNA prepared from *Euplotes octocarinatus* cells as described previously (Liang *et al.* 1994) or 25 µl of cell lysates. For preparations of *Euplotes* lysates as DNA-templates for RAPD polymerase chain reactions, test ciliates were first transferred from culture medium to sterile double distilled water or sterile artificial marine water. A further transfer to 5 ml of sterile water was performed in order to remove any contamination. Individual ciliates were isolated in a volume of 10 µl with the help of a dissecting microscope and transferred into PCR-tubes. Samples of 10 µl volume without ciliates were processed as controls. To each tube was added 90 µl of lysis buffer and the mixtures were incubated at 56°C for 60 min followed by 95°C for 15 min. Afterwards the ciliate lysates and controls were stored at-20°C (Kusch and Heckmann 1996, Brünen-Nieweler *et al.* 1998).

Amplifications by PCR were carried out in a total volume of 50 µl containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 µM of one oligonucleotide primer (Table 1) and 2.5 U of Taq DNA polymerase (Promega). A total of 45 oligonucleotide primers were tested initially, the catalogue numbers of which were as follows: E1, E2, No. 9 and No. 832 (TaKaRa Bio. Co., Japan); S21-S40, S101-S120 and S2001 (Sangon Bio. Co., Canada). The amplification mixtures were covered with 25 µl of mineral oil and placed in a PCR thermal cycler. For amplification the reaction mixtures were denatured at 94°C for 5 min, followed by the first 5 cycles consisting of denaturation for 30 s at 94°C, primer annealing for 30 s at 35°C, and extension for 1 min at 72°C. The subsequent 35 cycles comprised denaturation for 30 s at 94°C, primer annealing for 30 s at 40°C, and extension for 1 min at 72°C. Cycling was followed by a final extension step for 5 min at 72°C. PCR products (10 µl), along with a DNA molecular weight marker, were run on a 1.5% agarose gel. Three repetitions of the PCR reaction were performed in order to assess the reproducibility of the data.

The requisite concentration of DNA template for RAPD fingerprinting was determined by carrying out the PCR reaction with different numbers of *E. vannus* cells. To PCR-tubes containing 25 µl of lysis buffer was added 1, 3, 5, 10, or 20 cells of *E. vannus* respectively, which provided the source DNA template for the PCR reaction. The resulting PCR product was run on a 1.5% agarose gel.

**Morphometric data**. Data for the morphometric analysis was obtained from the literature as indicated in Tables 2 and 3. In addition

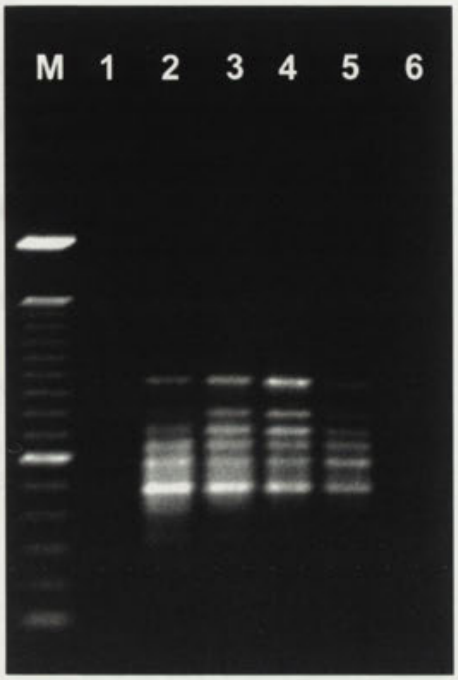


**Fig. 1.** Gradient of DNA template of *Euplotes vannus* RAPD fingerprinting with primer E1. Lanes: M - 100 bp marker, 1 - control without cells, 2 - 1 cell, 3 - 3 cells, 4 - 5 cells, 5 - 10 cells, 6 - 20 cells

to the 11 morphometric characters listed, two others were also included: the dorsal ridges (present *vs.* absent) and macronucleus shape. No character weighting was included.

**Data analysis.** The Cluster program in Statistica software (ver 5.1, StatSoft, Inc. 1984-1996) was used both for calculating the phylogenetic relationships among the six taxa and for tree construction. The band-sharing index (D) was calculated for all possible pairwise comparisons. The band-sharing index for two individuals is given by the formula: $D = 2N_{AB} / (N_A+N_B)$ where $N_A$ and $N_B$ are the number of bands scored in ciliates A and B respectively, and $N_{AB}$ is the number shared by both (Wetton *et al.* 1987).

## RESULTS AND DISCUSSION

### RAPD fingerprinting using different numbers of cells of *E. vannus*

The results of RAPD fingerprinting using different numbers of *E. vannus* cells are shown in Fig. 1. The control solution (without DNA template, lane 1) did not produce bands. Lanes 2-5, representing original inocula of 1, 3, 5, and 10 cells respectively, each had 8 bands. There were no bands in lane 6 (20 cells), possible reasons for which include; (i) the concentration of DNA template was too high; (ii) other cellular constituents (i.e. proteins, lipids, etc.) were present in excessive amounts; (iii) a combination of these two factors.

The most intense bands were found in lanes 3 and 4 (3-5 cells). Although the intensities of several amplified segments varied with the concentration of template DNA, the positions of bands did not change relative to one another indicating that our results had satisfied the criterion of reproducibility. The fact that a single cell was

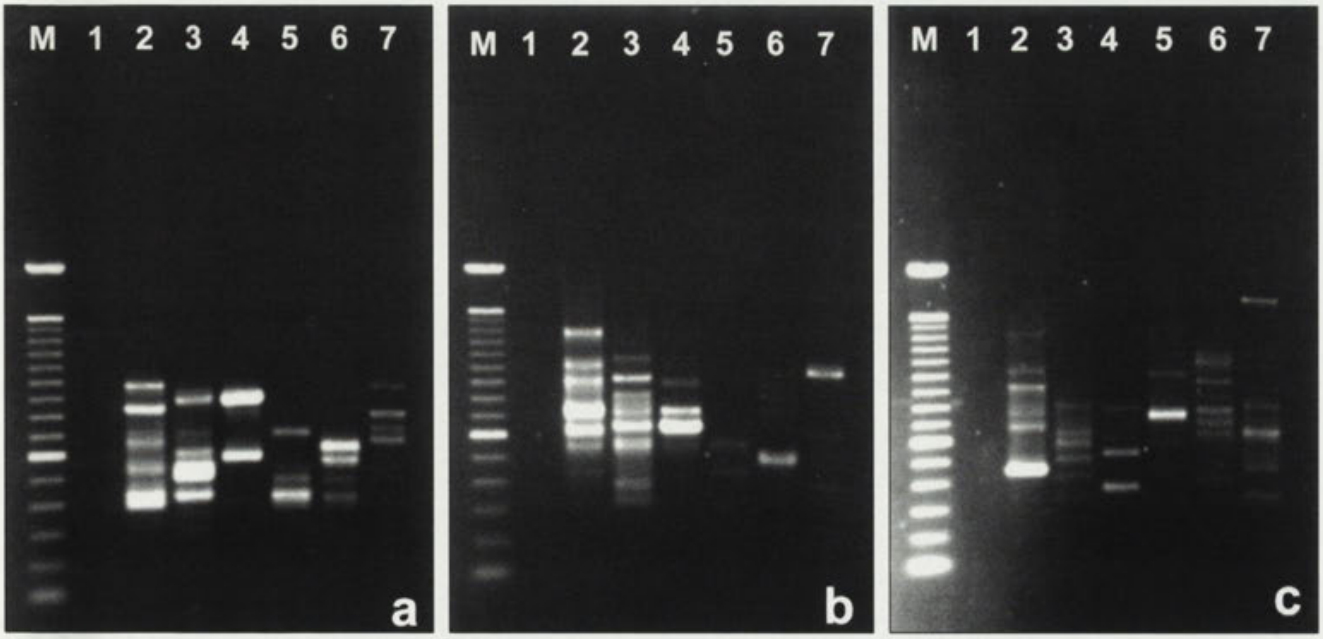


**Fig. 2.** RAPD fingerprinting of six *Euplotes* species with primers E1 (**a**), No.832 (**b**) and S101 (**c**). Lanes: M -100 bp marker, 1 - control without cells, 2 - *E. woodruffi*, 3 - *E. eurystomus*, 4 - *E. octocarinatus*, 5 - *E. charon*, 6 - *E. minuta*, 7 - *E. vannus*

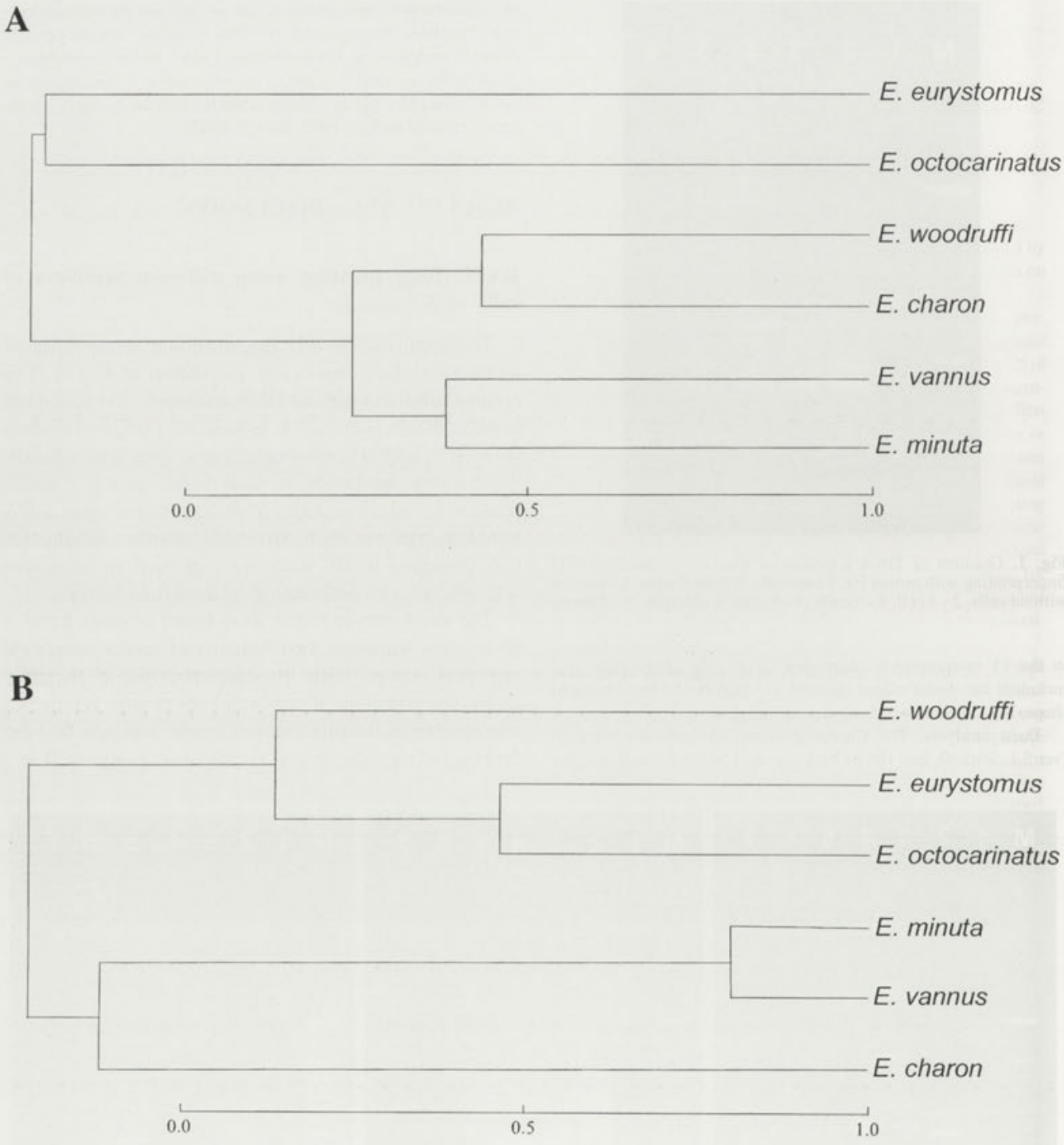


**Fig. 3. A** - phylogenetic dendogram of six *Euplotes* species, based on RAPD fingerprinting. **B** - phylogenetic dendogram of the same six species, based on morphometric characters. Both dendograms prepared using UPGMA in PHYLIP (ver 3.57c, Felsenstein 1995). The calculated mutation distances are indicated for each branch

**Table 2.** Morphometric characterization of *Euplotes woodruffi* (1st line), *E. eurystomus* (2nd line), *E. octocarinatus* (3rd line), *E. charon* (4th line), *E. minuta* (5th line) and *E. vannus* (6th line). Data are based on protargol impregnated specimens. Measurements in µm. AZM - adoral zone of membranelles, Max - maximum value, Min - minimum value, n - sample size, SD - standard deviation, SE - standard error of arithmetic mean, CV - coefficient of variation in %, (-) - data not available. Data sources: *E. woodruffi* (after Song and Bradbury 1997); *E. eurystomus*, present paper; *E. octocarinatus* (after Carter 1972); *E. charon* and *E. vannus* (after Song and Packroff 1996/97); *E. minuta* (after Song and Wilbert 1997)

| Character | Min | Max | Mean | SD | SE | CV | n |
|---|---|---|---|---|---|---|---|
| Length of body | 105 | 134 | 117.0 | 5.69 | 1.58 | 4.9 | 13 |
| | 108 | 136 | 117.9 | 9.34 | 2.33 | 7.9 | 16 |
| | 61.5 | 99 | 80.0 | - | - | - | - |
| | 75 | 98 | 85.1 | 7.33 | 1.83 | 8.6 | 16 |
| | 45 | 78 | 57.2 | 8.37 | 2.09 | 14.6 | 16 |
| | 96 | 135 | 124.2 | 8.69 | 2.17 | 7.0 | 16 |
| Body width | 84 | 93 | 87.7 | 2.59 | 0.72 | 3.0 | 13 |
| | 64 | 100 | 81.6 | 8.89 | 2.22 | 10.9 | 16 |
| | 33 | 66 | 50.0 | - | - | - | - |
| | 67 | 90 | 74.6 | 8.12 | 2.03 | 10.9 | 16 |
| | 31 | 54 | 40.1 | 5.76 | 1.44 | 14.3 | 16 |
| | 61 | 78 | 69.2 | 5.18 | 1.30 | 7.5 | 16 |
| Length of buccal field | 84 | 90 | 86.7 | 1.97 | 0.55 | 2.3 | 13 |
| | 68 | 88 | 79 | 5.93 | 1.48 | 7.5 | 16 |
| | 33 | 55.5 | 50.1 | - | - | - | - |
| | 61 | 77 | 66.3 | 4.60 | 1.15 | 6.9 | 16 |
| | 35 | 43.0 | 38.4 | 2.13 | 0.53 | 5.5 | 16 |
| | 84 | 108 | 98.1 | 6.99 | 1.75 | 7.1 | 16 |
| No. of frontoventral cirri | 9 | 9 | 9 | 0 | 0 | 0 | 18 |
| | 9 | 9 | 9 | 0 | 0 | 0 | 16 |
| | 9 | 9 | 9 | - | - | - | - |
| | 10 | 10 | 10 | 0 | 0 | 0 | 16 |
| | 10 | 10 | 10 | 0 | 0 | 0 | 25 |
| | 10 | 10 | 10 | 0 | 0 | 0 | 16 |
| No. of transverse cirri | 5 | 5 | 5 | 0 | 0 | 0 | 18 |
| | 5 | 5 | 5 | 0 | 0 | 0 | 16 |
| | 5 | 5 | 5 | - | - | - | - |
| | 4 | 6 | 5.1 | 0.44 | 0.11 | 8.7 | 16 |
| | 5 | 5 | 5 | 0 | 0 | 0 | 25 |
| | 5 | 5 | 5 | 0 | 0 | 0 | 16 |
| No. of caudal cirri (MC and CC) | 4 | 4 | 4 | 0 | 0 | 0 | 20 |
| | 4 | 5 | 4.1 | 0.25 | 0.06 | 6.2 | 16 |
| | 4 | 4 | 4 | - | - | - | - |
| | 4 | 7 | 4.4 | 0.63 | 0.16 | 14.2 | 16 |
| | 4 | 4 | 4 | 0 | 0 | 0 | 25 |
| | 4 | 6 | 4.5 | 0.51 | 0.13 | 11.6 | 16 |
| No. of dorsal kineties | 10 | 10 | 10 | 0 | 0 | 0 | 13 |
| | 8 | 9 | 8.6 | 0.50 | 0.13 | 5.8 | 16 |
| | 8 | 8 | 8 | - | - | - | - |
| | 9 | 10 | 9.5 | 0.52 | 0.13 | 5.4 | 16 |
| | 7 | 9 | 8.6 | 0.63 | 0.16 | 7.4 | 16 |
| | 9 | 10 | 9.8 | 0.40 | 0.10 | 4.1 | 16 |
| No. of basal bodies in mid-dorsal kineties | 23 | 28 | 24.5 | 1.45 | 0.40 | 5.9 | 13 |
| | 17 | 25 | 20.9 | 2.43 | 0.54 | 11.6 | 20 |
| | *ca* 18-21 | | | | | | |
| | 10 | 25 | 22.3 | 1.92 | 0.48 | 8.6 | 16 |
| | 9 | 11 | 10.1 | 0.81 | 0.20 | 7.9 | 16 |
| | 16 | 21 | 18.3 | 1.34 | 0.27 | 7.3 | 24 |
| No. of membranelles in AZM | 57 | 64 | 60.1 | 1.94 | 0.65 | 3.2 | 9 |
| | 42 | 54 | 46.2 | 3.36 | 0.87 | 7.3 | 15 |
| | 36 | 42 | 38 | - | - | - | - |
| | 51 | 60 | 54.5 | 3.88 | 1.17 | 7.1 | 11 |
| | 33 | 41 | 36.6 | 2.15 | 0.54 | 5.8 | 18 |
| | 53 | 66 | 57.1 | 4.46 | 1.11 | 7.8 | 16 |

**Table 3.** Pattern of silverline system on dorsal side, and the biotope, of six *Euplotes* species (after Curds 1975)

| | Single-*vannus* | Double-*eurystomus* | Double-*patella* | Habitat |
|---|---|---|---|---|
| *E. woodruffi* | + | | | Freshwater |
| *E. eurystomus* | + | | | Freshwater |
| *E. octocarinatus* | | | + | Freshwater |
| *E. charon* | | + | | Marine |
| *E. minuta* | + | | | Marine |
| *E. vannus* | + | | | Marine |

found to produce sufficient DNA template for the RAPD reaction to work successfully shows that this method can be used without the need to cultivate the organism.

## Evolutionary relationships inferred from RAPD fingerprinting

As a result of the findings reported above, 3-5 cells from each clonal culture were used to provide the DNA template for the investigations of DNA polymorphism and genetic diversity. Three out of 45 oligonucleotide primers tested generated informative bands; altogether 10 bands were scorable with primer E1, 13 bands with primer No.832, and 19 bands with primer S101 (Fig. 2). The same band patterns were produced for each taxon on each of three separate occasions thereby verifying the reproducibility of these results. Other primers gave less discernible or no band patterns. The control without template DNA amplified nothing

An examination of Fig. 2 shows that, for each of the three primers used, there are significant differences in the banding patterns among all six taxa. This indicates that even morphologically very similar species, e.g. *E. vannus* and *E. minuta,* can be clearly separated using RAPD fingerprinting. From Table 4 it can be seen that genetically (i.e. as measured by the band sharing index, D), the most closely related taxa were *E. vannus* and *E. minuta* (D = 0.5854) followed by *E. vannus* and *E. woodruffi* (D = 0.5455); *E. charon* was relatively distantly related to the other species, particularly *E. octocarinatus* (D = 0.3571). Thus, the range of the band sharing indices among the six taxa was *ca* 0.36-0.59. This compares with the range of 0.38-0.46 reported by Kusch and Heckmann (1996) in their investigation of three species of *Euplotes*.

Based on the results of the RAPD fingerprinting a phylogenetic dendogram was constructed (Fig. 3A). According to this dendogram, the six taxa grouped into two identifiable clusters; *E. vannus-minuta-charon-woodruffi*, and *E. eurystomus-octocarinatus*. Within the first cluster there were two clades, one formed by *E. minuta* and *E. vannus*, the other by *E. woodruffi* and *E. charon*. By contrast, *E. eurystomus* and *E. octocarinatus* were only distantly related, both to each other and to *E. minuta* and *E. vannus* (Fig. 3A). This was somewhat unexpected and probably erroneous because these four species have several important morphological characters in common (viz. patterns of silverline system and infraciliature) and could reasonably be expected to group together. A possible explanation for this is that the molecular data derived from RAPD fingerprinting are largely dependent on the choice of primer used. In this case, entirely different banding patterns were obtained for each taxon according to which of the three primers was used (see Fig. 2a,b,c). Furthermore, there were very few similarities between the banding patterns for any of the taxa studied, regardless of the primer used, suggesting that there is little justification for supposing that their phylogenetic relationships could be inferred from the RAPD fingerprinting data.

## Morphometric analysis

The phylogenetic dendogram based on morphometric characters is shown in Figure 3B and reveals two main clusters: *E. vannus-minuta-charon*, and *E. eurystomus-octocarinatus-woodruffi*. Among these, *E. minuta* and

**Table 4.** Band sharing index (D) of RAPD fingerprinting for interspecies genetic diversity of six *Euplotes* species

| | *E. woodruffi* | *E. eurystomus* | *E. octocarinatus* | *E. charon* | *E. minuta* | *E. vannus* |
|---|---|---|---|---|---|---|
| *E. woodruffi* | - | 0.4390 | 0.5143 | 0.5143 | 0.5128 | 0.5455 |
| *E. eurystomus* | 0.4390 | - | 0.5294 | 0.4706 | 0.5263 | 0.5116 |
| *E. octocarinatus* | 0.5143 | 0.5294 | - | 0.3571 | 0.5000 | 0.4324 |
| *E. charon* | 0.5143 | 0.4706 | 0.3571 | - | 0.3750 | 0.4324 |
| *E. minuta* | 0.5128 | 0.5263 | 0.5000 | 0.3750 | - | 0.5854 |
| *E. vannus* | 0.5455 | 0.5116 | 0.4324 | 0.4324 | 0.5854 | - |

*E. vannus* were the most closely related, followed by *E. octocarinatus* and *E. eurystomus*. Although *E. charon* was revealed as an isolated branch and sister taxon to the *E. minuta-vannus* group, the support for this relationship did not appear to be very high. Thus, the dendogram based on morphological data differs significantly from that based on RAPD analysis, particularly with respect to the relative positions on the trees of *E. woodruffi* and *E. charon* (Fig. 3A, B).

Morphological differences among *Euplotes* spp. have previously been analyzed by Borror and Hill (1995) who recognized four main groups and, as a result, proposed the quadripartition of the genus. Unsurprisingly, the phylogenetic tree based on morphological characters presented here reflects Borror and Hill's (1995) groupings for the six taxa in question. However, because of the small number of taxa included in the present study, it was not possible to reach any conclusion as to the taxonomic level these groupings represent, i.e. generic or subgeneric level.

## Conclusions

As this study has demonstrated, RAPD fingerprinting is potentially a very useful technique for the separation of morphologically similar taxa. For example, *E. minuta* and *E. vannus*, or *E. eurystomus* and *E. octocarinatus*, have relatively minor morphologically differences but could be clearly separated by RAPD fingerprinting (Fig. 2). This study also confirms a previous report that RAPD fingerprinting can be successfully applied even when only single cells are available (Kusch and Heckmann 1996). However, this technique seems to be less useful for determining phylogenetic relationships at species level since the banding patterns vary considerably according to the primer used (Fig. 2). Furthermore, in the present study the high degree is dissimilarity among the banding patterns rendered it difficult to make any inference about the phylogenetic interrelationships among the six taxa investigated. Nevertheless, the application of molecular biological techniques such as enzyme electrophoresis, DNA/DNA-hybridizations, and SSUrRNA sequence comparisons (Schlegel *et al.* 1988, 1991; Orias *et al.* 1991; Jerome and Lynn 1996; Kusch and Heckmann 1996; Lynn *et al.* 1999), in parallel with the continued use of morphological characters, should facilitate a better understanding of phylogenetic relationships among the ciliated protozoa in general, and euplotids in particular.

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# Molecular Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* from Stool and Culture Samples Obtained from Polish Citizens Infected in Tropics and in Poland

**Przemysław MYJAK[1], Józef KUR[2], Halina PIETKIEWICZ[1], Andrzej KOTŁOWSKI[1], Wacław NAHORSKI[1], and Beata SZOSTAKOWSKA[1]**

**[1]Institute of Maritime and Tropical Medicine, Gdynia; [2]Department of Microbiology, Technical University of Gdańsk, Poland**

**Summary.** The examinations were carried out on stool and serum samples obtained from 38 Polish citizens infected with *E. histolytica sensu lato*. Prevalence of infection (according microscopic examinations) was 0.63% in the Polish who come back from abroad and about 0,19% in persons who did not leave our country. The investigations were performed with isoenzyme analysis and polymerase chain reaction with the use of specific (Psp, NPsp) and (p11+p12, p13+p14) primers. Serodiagnostic examinations were done with antigen produced from *E. histolytica* HK-9 axenic strain. The examinations revealed that in 31 cases the amoebae belonged to nonpathogenic *E. dispar*, in 4 to pathogenic *E. histolytica*, among this one indigenous case; and in two cases were mixed infections. In one case we neither got DNA amplification products nor cultured amoebae. However, from 25 persons we obtained amoebae cultures helping in identification the amoebae zymodemes. The prevailing zymodeme was nonpathogenic zymodeme I. The PCR results were in agreement with isoenzymatic and serodiagnostic examinations.

**Key words:** DNA isolation, *Entamoeba dispar*, *E. histolytica*, genotype, PCR, phenotype, restriction enzyme, serology, zymodeme.

**Abbreviations:** CIEP - counter immunoelectrophoresis test, GPI - glucose-phosphate isomerase E.C. 5.3.1.9., HK - hexokinase E.C. 2.7.1.1, IFA - indirect fluorescent antibody test, IHA - indirect hemagglutination test, ME - malic enzyme E.C. 1.1.1.40, PCR - polymerase chain reactions, PGM - phosphoglucomutase E.C. 2.7.5.1, RAPD - Random Amplified Polymorphic DNA assay.

## INTRODUCTION

Very shortly after the first description of amoebic dysentery (Lösh 1875) it was noticed that clinical symptoms only occurred in some of persons infected with *Entamoeba histolytica*. Therefore as far back as in 1925 a concept of the existence of two morphologically identical amoeba species was formulated (Brumpt 1925). This idea, however, was not accepted then. It was not until 1993 that Diamond and Clark (1993) gave the ultimate redescription of *E. histolytica* species with morphologically identical cysts, leaving the name *E. histolytica* (Schaudinn, 1903) for the pathogenic strains and restoring the specific name *E. dispar* for nonpathogenic strains, previously proposed by Brumpt (1925).

Address for correspondence: Przemysław Myjak, Department of Tropical Parasitology, Institute of Maritime and Tropical Medicine, 81-519 Gdynia, ul. Powstania Styczniowego 9 b, Poland; Fax: +48 58 6223354; E-mail: pemyjak@immt.gdynia.pl

The infection with *E. histolytica sensu lato* is one of the most common parasitic infections in humans worldwide. It is estimated that about 10% of the world population (Guerrant 1986) is infected with this parasite. Clinical symptoms are observed in 10% of the infected people only, which is related with *E. histolytica* infection, 90% of the cases are asymptomatic, which is most frequently attributed to *E. dispar* infection. Nonetheless, since in some *E. histolytica* infection cases the symptoms may not appear at a certain moment, the number of infections is expected to be markedly higher. People infected with *E. dispar* do not require medical treatment (WHO 1997). It is therefore of vital importance to differentiate these two amoebic species which are morphologically identical.

Hence, other methods that would not be based on the morphology of the parasite should be worked out to detect and/or differentiate amoeba species.

In view of the above, isoenzyme analysis, used already for 20 years (Sargeaunt *et al.* 1978, Farri *et al.* 1979), permit to differentiate *E. histolytica sensu lato* strains as pathogenic and nonpathogenic ones, at present identified with *E. histolytica* and *E. dispar*, respectively.

From the beginning of the present decade, many efforts have been made to employ the technique of polymerase chain reaction (PCR) in the differentiation of these species (Acuna-Soto *et. al.* 1993, Katzwinkel-Wladarsch *et al.* 1994, Novati *et al.* 1996, Britten *et al.* 1997, Troll *et al.* 1997, Walderich *et al.* 1997, Haque *et al.* 1998).

In addition, detection of amoebae coproantigens is very helpful; introduction of monoclonal antibodies specific for *E. histolytica* and *E. dispar* allows their differentiation (Haque *et al.* 1995, 1998, Jackson and Rawdin 1996, Mirelman *et al.* 1997).

The aim of the present study embraced: (1) molecular differentiation of *E. histolytica* and *E. dispar* in Polish citizens returning from tropical and subtropical countries, as well as in those who did not leave Poland or Europe and (2) the determination of the prevalence of *E. histolytica sensu lato* genotypes and phenotypes in Poland.

In our previous article (Myjak *et al.* 1998) we presented the results of PCR amplifications directly from the faeces of the persons examined. In this work we compare the results obtained from the larger number of examinations both PCR directly from faeces and PCR and isoenzyme analysis from amoebae cultures *in vitro*.

## MATERIALS AND METHODS

**Sample material.** In the years 1995-1998, 4270 Polish citizens who returned from the tropical and subtropical regions were examined in the Institute of Maritime and Tropical Medicine, according scheme using since many years (Zwierz *et al.* 1975). Twenty-seven persons (0.63%) were infected with *E. histolytica s. l.* Amoebae were also found in 6 (0.19%) persons who did not leave either Poland or Europe. Samples from 5 infected persons were obtained from another laboratory. Stool and serum specimens from those patients were obtained and examined.

**Cultivation of amoeba.** Three kinds of medium were used: (1) liquid medium PAHM (Myjak 1967), (2) bi-phasic Robinson's medium (Robinson 1968), and (3) solid medium MA (Myjak 1971).

**Serological tests.** Indirect hemagglutination test (IHA) and other tests were performed according to the standard procedure (Myjak *et al.* 1979) with an antigen obtained in our laboratory from *E. histolytica* HK-9 axenic strain.

**Isoenzymatic determination.** Isoenzyme analyses were performed when a stable and sufficient amoeba culture was obtained on PAHM or Robinson's medium. The lysate of amoebae and isoenzymatic examinations of 4 enzymes (HK /E.C. 2.7.1.1./; PGM /E.C. 2.7.5.1 /; GPI /E.C. 5.3.1.9/; ME /E.C. 1.1.1.40/); were performed using the method described by Sargeaunt *et al.* (1978) and Farri *et al.* (1979).

### Polymerase chain reaction (PCR)

**Isolation of DNA.** DNA was isolated from: (1) the in vitro culture and, in some cases, from the stool, using a Genomic DNA Prep Plus kit (A&A Biotechnology, Gdynia, Poland) (Myjak *et al.* 1997); (2) from cysts and/or trophozoites present in the stool, in accordance with the procedure described by da Silva *et al.* (1996), with the final step of DNA purification carried out by means of a DNA Clean-Up kit (A&A Biotechnology, Gdynia).

**Primers.** Specific primers used for *E. histolytica* (Psp5 and Psp3) and for *E. dispar* (NPsp5 and NPsp3) were described by Clark and Diamond (1992). Moreover, in the comparative studies, primers (p11+p12 for *E. histolytica* and p13+p14 for *E. dispar* respectively) complementary to the sequence of DNA encoding 30 kDa protein were used, described by Tachibana *et al.* (1991). For PCR fingerprinting, a 10-nucleotide RAPD 3 primer was used: 5'- GTA GAC CCG T.

**DNA amplification.** 2 µl of genomic DNA was added to the reaction mixture: 5 µl of PCR buffer (10 x concentrated), 5 µl of dNTP mixture (concentration of each dNTP was 2.5 mM), 2 µl of each primer (10 µM) and 33 µl of water. The mixture was supplemented with 1 U (1 µl) of *Taq* DNA polymerase (Shark-2, DNA - Gdańsk, Poland) or Ampli Taq® (Perkin-Elmer; 1,25 U = 0.25 µl).

Amplification was carried out in a GeneAmp PCR System 2400 (Perkin-Elmer) thermal cycler according to the following scheme: initial denaturation for 2 min at 94°C, followed by 35 cycles of denaturation for 1 min at 94°C, 1.5 min of annealing at 55°C, the extension for 2 min at 72°C, and the final extension step for 5 min at 72°C. All the PCR were carried out in a 50 µl volume.

For comparison, the amplifications with primers p11+p12 and p13+p14 were carried out according to the thermal profile of Tachibana *et al.* (1991). In RAPD-PCR, the amplification profile was as the profile with primers Psp and NPsp, but the annealing was performed for 1.5 min at 30°C.

**Detection of PCR products and restriction fragments.** Some of the amplification products with primers Psp and NPsp were additionally analysed following their digestion with the restriction endonuclease *Sau*96I (Promega), using the manufacturer's recommended procedure. The specific PCR products and restriction fragments obtained by enzyme digestion were separated electrophoretically on a 2% agarose gel (Sigma A-9539) while obtained with RAPD 3 primer on 8 % polyacrylamide gel using standard procedures. The gels were stained with ethidium bromide, visualised by UV and photographed.

## RESULTS

**Isoenzyme analyses.** *E. histolytica sensu lato* was detected in the stool samples from 37 persons. In 28 out of those 37 cases (75,7%), amoebae were found in cultures, from which 25 were stable (67,6%), thus enabling isoenzyme analyses to be performed. From among these 25 isolates, 3 were classified as pathogenic and 22 as nonpathogenic according to the obtained zymodemes (Table 1).

**Results of serological tests.** All sera collected from persons infected with *E. dispar* were IHA negative except for two with lower IHA titre (1:81-1:243). Five of 6 sera from persons infected with *E. histolytica* were IHA (Table 1), CIEP and IFA positive.

### PCR determinations

**PCR determinations from short-lived culture.** The PCR was carried out with DNA isolated from a short-lived culture (1-2 subculture) of amoeba (n = 28). In two persons *E. histolytica* was detected, one was suspected of a mixed invasion of *E. histolytica* and *E. dispar*, and in 25 cases *E. dispar* was diagnosed (Table 1).

**PCR results obtained directly from the clinical samples.** Positive DNA amplification results were directly obtained in 36 (97,3%) stool samples out of 37 persons (with *E. histolytica s. l.* found in stool) examined. In 3 persons, *E. histolytica* was detected. DNA amplifications with *E. histolytica* and *E. dispar* primers were achieved in two cases, whereas in 31 persons, only with *E. dispar* primers were detected (Table 1, Fig. 1A).

In patient (No. 340) with amoeba detected in histological preparations of the intestine, the DNA template isolation from these samples following standard removal of paraffin, xylene and alcohol was unsuccessful.

In one case examined, indicating dysenteric amoebiasis at first, *E. dispar* was recognised by PCR and isoenzymatic examinations, while IHA was negative. Therefore, the stool was cultivated on bacterial media and the *Shigella* bacteria then harvested, turned out to be the cause of the dysentery.

**Digestion of PCR products with *Sau*96I.** In the samples of amplified DNA, 23 of the samples investigated (stool, culture) were additionally verified by digesting the PCR products with restriction enzyme *Sau*96I. As the result of enzymatic digestion of the PCR products obtained from 14 clinical samples examinations, two bands of about 740 and 140 bp were displayed, characteristic of *E. dispar*, whereas in two samples (and in two control samples of *E. histolytica* HK-9 and 200:NIH), of the electrophoretic patterns displayed, there was only one nondigested band of 876 bp, characteristic of *E. histolytica* (data not shown). In three samples from two patients (Nos. 376 and 388) in which DNA amplifications were positive with two pairs of primers, digestion of PCR products with restriction enzyme *Sau*96I gave one 876 bp band with *E. histolytica* primer product and two bands (about 740 and 140 bp) with *E. dispar* primer product, which illustrated mixed infections (Fig. 1B).

**Identity between *E. dispar* isolates.** Two *E. dispar* isolates (Nos. 324, 325) were collected from a married couple, No. 325 from the wife who did not leave Poland. Both isolates belonged to nonpathogenic zymodeme I (Table 1). The electrophoretic profiles obtained with RAPD 3 primer for above two isolates were identical, so, these isolates were considered to represent the same strain. The bacteria isolated from their culture gave different profiles, although some of the bands showed the same size as that obtained with amoeba (Fig. 1C).

The same RAPD-PCR method was successfully used for genotyping of another *E. dispar* and *E. histolytica* isolates. The results show that isolates were genetically unrelated regarding the number of bands and the size of DNA fragments (Fig. 1C for *E. dispar* No 328 isolate).

**PCR sensitivity.** The examinations were performed with DNA isolated from trophozoites from an axenic culture of *E. histolytica* strain HK-9 and polyxenic culture of *E. dispar* isolate No. 350. The PCR sensitivity was determined by DNA isolations from 1000, 100, 10, 5 and one amoebae followed by PCR amplification with *E. histolytica* (Psp5+Psp3) and *E. dispar* (NPsp5+NPsp3) specific primers. The analysis performed has shown that the amplification of amoebae DNA with specific primers for both amoeba species, a number of 5 trophozoites in the sample was sufficient (data not shown).

**Table 1.** Laboratory and clinical examinations of patients infected with *Entamoeba histolytica sensu lato*

| sample | | examinations | | | | | | symptoms | location / time |
|---|---|---|---|---|---|---|---|---|---|
| | | micros-copic | serology | culture *in vitro* | zymodeme | PCR from: | | | |
| number | n | | (IHA) | | | stool | culture | | |
| 329 | 1 | c | - | - | ND | E. d. | ND | none | Paraguay, 92-95 |
| 318 | 3 | c, t | - | + | np. ? | E. d. | E. d. | none | Zambia, 87-94 |
| 325• | 2 | c, t | - | + | np. I | E. d. | E. d | none | South Africa, 77 Argentina, 78 |
| 337 | 1 | c | - | + | np. I | E. d. | E. d. | none | S. America, Africa, Asia, all time |
| 327 | 2 | c, t | - | +- | ND | E. d. | E. d. | none | Cameroon, 82-95 |
| 362 | 1 | c, t | - | + | np. ? | E. d. | E. d. | none | UEA, 95 |
| 363 | 3 | c | - | + | np. I | E. d. | E. d. | none | Zaire, 93-96 |
| 371 | 3 | c | - | +- | ND | E. d. | E. d. | none | Ghana, 91-96 |
| 89 | 3 | c, t | - | + | np. I | E. d. | E. d. | none | Thailand, Malaysia, 3-5.97 |
| 372•• | 1 | c | - | + | np. I | E. d. | E. d. | none | Turkey, Morocco, Mexico Gulf, 8.96-2.97 |
| 373•• | 1 | c | - | +- | ND | E. d. | E. d. | none | Poland |
| 374 | 3 | c, t | - | + | np. ? | E. d. | E. d. | none | Uganda, 94-97 |
| 381 | 2 | c | - | - | ND | negative | ND | none | RSA, 91-97 |
| 386 | 1 | c | - | + | np. I | E. d. | E. d. | none | Poland |
| 392 | 1 | c, t | - | + | np. I | E. d. | E. d. | none | Poland |
| 403 | 1 | c | 1:81 | - | ND | E. d. | ND | none | Libya, 96-98 |
| 397 | 1 | c | - | - | ND | E. d. | ND | none | America, all time |
| 407 | 1 | c | - | + | np. ? | E. d. | E. d. | none | Congo, 73-98 |
| 409 | 1 | c, t | - | + | np. I | E. d. | E. d. | none | India, 98 - 5 months |
| 410 | 2 | c | - | + | np. ? | E. d. | E. d. | none | Middle East, 7.97-5.98 |
| 414 | 2 | c | - | - | ND | E.d. | ND | none | Congo, 90-99 |
| 396 | 1 | c, t | 1:243 | - | ND | E. h. | ND | none | Congo, 87-96 |
| 404 | 3 | c, t | - | + | np. I | E. d. | E. d. | n.d.c. | Turkey, 98 - 2 weeks |
| 395 | 1 | c | - | - | ND | E. d. | ND | n.d.c. | Egypt, 12.97 |
| 330 | 4 | c, t | - | + | np. I | E. d. | E. d. | n.d.c. | Italy, 94, Spain, 95 |
| 324• | 2 | c, t | - | + | np. I | E. d. | E. d. | n.d.c. | Poland |
| 326 | 2 | c, t | - | + | np. I | E. d. | E. d. | n.d.c. | Cameroon, 90-95 |
| 328 | 1 | c, t | - | + | np. XVII | E. d. | E. d. | n.d.c. | West Africa, 4-9.95 |
| 383 | 3 | c, t | - | + | np. I | E. d. | E. d. | n.d.c. | Cameroon, 92-96 |
| 402 | 1 | c | - | - | ND | E. d. | ND | n.d.c. | India, 98, 1 month |
| 401 | 1 | c | 1:243 | + | np. I | E. d. | E. d. | n.d.c. | Chad, 97 |
| 350 | 3 | c | - | + | np. I | E. d. | E. d. | n.d.c. | Bahrain, 1-5.95 |
| 388 | 2 | c | - | - | ND | E. h/E. d | ND | n.d.c. | Rwanda, 80-97 |
| 384 | 2 | c, t | 1:729 | + | p. II or XIV | E. h. | E. h. | n.d.c. | Cameroon, 89-97 |
| 376 | 3 | c | 1:2187 | + | p. II | E. h/E. d | E. h./ E.d. | n.d.c. | India, North Africa, 92-97 |
| 351 | 1 | c | - | +* | np. I | E. d. | E. d. | dysentery | Rwanda, 89-96 |
| 333 | 4 | c, t | 1:6561 | + | p. XIV | E. h. | E. h. | liver abscess | Angola, Cameron, Venezuela, Peru, 1995 |
| 340 | 2 | - t, in tissues | 1:6561 | ND | ND | negative** | ND | liver abscess, intestinal ulcers | Poland *** |

•; •• = marriage, * = *Shigella* isolated from stool, ** = stool was examined after therapy, *** = patient had a contact with Liberian ship cook in the shipyard, c = cysts, t = trophozoites without erythrocytes, n.d.c. = nondysenteric colitis, - = negative, + = positive, +- = short lived culture, np. = nonpathogenic zymodeme, p = pathogenic zymodeme, ? = zymodeme number not determined, ND = not done, E.h. = *E. histolytica*, E.d. = *E. dispar*

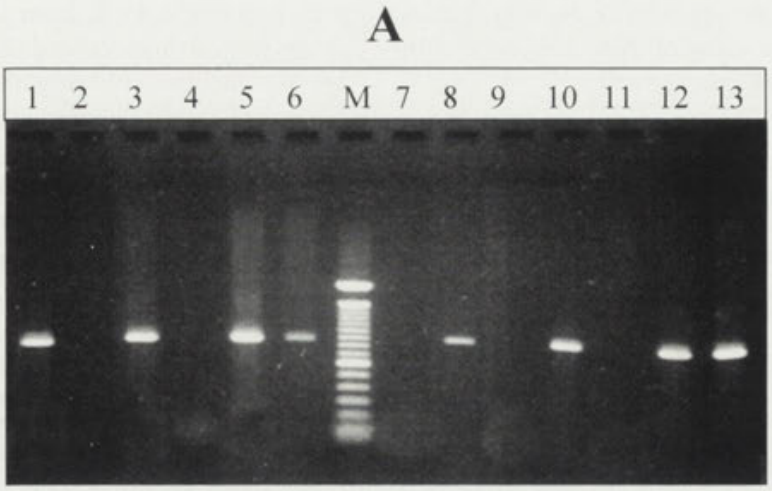
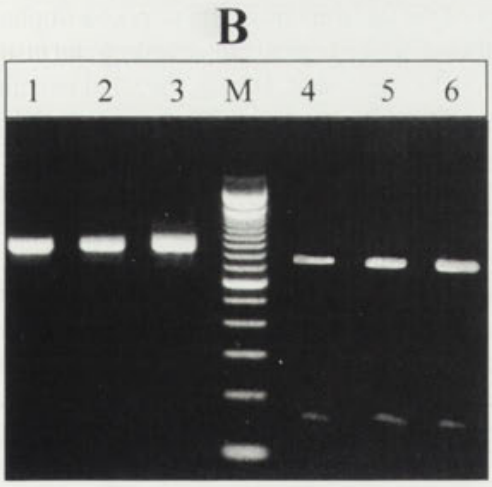
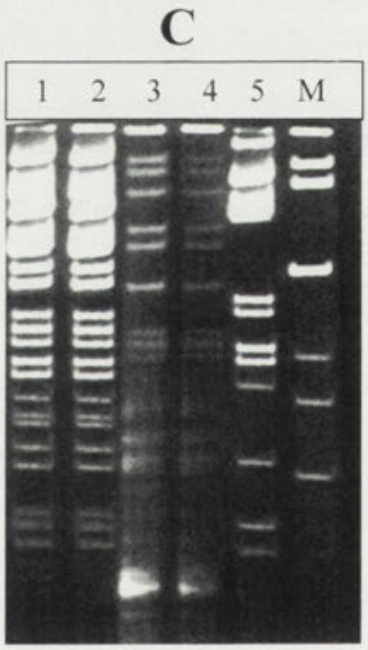



**Fig. 1.** PCR results. **A** - PCR amplification products obtained with *Entamoeba histolytica* Psp5+Psp3 primers (lanes: 1, 3, 5, 7, 9, 11) and *Entamoeba dispar* NPsp5+NPsp3 primers (lanes: 2, 4, 6, 8, 10, 12, 13). Lanes 1, 2 - *E. histolytica* 200: NIH; lanes 3, 4 - sample No 333 (*E. histolytica*); lanes 5, 6 - sample No 376 (*E. histolytica* and *E. dispar*); lanes 7, 8 - sample No 351 (*E. dispar*); lanes 9, 10 - sample No 383 (*E. dispar*); lanes 11, 12 - sample No 386 (*E. dispar*); lane 13 - sample No 389 - (*E. dispar*); lane M - 100 bp DNA ladder (Gibco-BRL).




**B** - RFLP analysis. Restriction endonuclease patterns of PCR products (876 bp) digested with *Sau*96I. Lane 1 - *E. histolytica* 200:NIH, lane 2 - sample No 333, lane 3 - sample No 376; all PCR products obtained with *E. histolytica* primers; lane 4 - sample No 376, lane 5 - sample No 386, lane 6 - sample No 389; all products obtained with *E. dispar* primers; lane M - 100 bp DNA ladder (Gibco-BRL).




**C** - PCR fingerprinting of DNA samples from marriage (Nos. 324 and 325) and control amoeba and bacteria isolates. Lane 1 - *E. dispar* isolate (No 324 - husband), lane 2 - *E. dispar* isolate (No 325 - wife), lane 3 bacteria from No 324 amoeba culture, lane 4 - bacteria from No 325 amoeba culture, lane 5 - *E. dispar* isolate (No 328), lane M - DNA markers (1008, 883, 615, 517, 466 and 396 bp)

**PCR results obtained with *E. histolytica* (p11+p12) and *E. dispar* (p13+p14) primers.** The usefulness of these primers for DNA amplification was verified on the example of 5 *E. histolytica,* 11 *E. dispar* and one mixed isolates (No. 376). The products of similar size (100 and 101 bp) were obtained with each of these primers, which

were complementary to the appropriate amoeba species DNA sequences, in the case of No. 376 only with *E. histolytica* primer (data not shown).

## DISCUSSION

The investigations carried out have shown that the methods used to isolate DNA from amoeba *in vitro* culture as well as directly from the stool are suitable for the PCR amplification of amoeba DNA, i.e. for differentiation of *E. histolytica* and *E. dispar*. The second method seems to be more effective.

The investigations have also confirmed that PCR is superior to isoenzyme analyses, since no satisfactory amoeba cultures could be obtained from as many as 32% of the patients, thus making impossible the isoenzyme examinations to be carried out. Walderich *et al.* (1997) and Haque *et al.* (1998) did not manage to obtain amoeba cultures in 43% and 8% of persons examined respectively, which also did not allow isoenzymatic determinations and PCR. On the other hand culturing allows for the detection of additional invasions (Haque *et al.* 1998).

In the case of the occurrence of nondysenteric colitis most persons (10/13) were infected by *E. dispar*. According to criteria established recently (WHO 1997), other causes of clinical symptoms observed in those persons should be investigated. Anand *et al.* (1997) reports that in some cases initially suspected of amoebic nondysenteric colitis, the irritable bowel syndrome, requiring specific treatment, was diagnosed most frequently.

It is interesting that in two cases (Nos. 376 and 388) the examinations using primers Psp and NPsp revealed a mixed infection with *E. histolytica* and *E. dispar*. A restriction analysis of the PCR products digested with *Sau*96I proved this observation (Fig. 1B). In view of this, we postulate that in the case of doubtful results, the restriction analysis of the products obtained should be performed. In the case of the isolate (culture) No. 376, the PCR with primers p11+p12 and p13+p14 and isoenzymatic examinations displayed the infection with one amoeba species only, i.e. *E. histolytica*. These differences in results may be explained by the supposition that p11+p12 and p13+p14 primers did not amplify *E. dispar* DNA from this amoeba isolate. The fact that isoenzyme analysis detected *E. histolytica* only, not *E. dispar*, suggests that the amoebae were in insufficient amount and a small number of *E. dispar* cells were eliminated by *E. histolytica*. PCR from templates obtained from cultures several times showed mixed infections. However, after six months culturing the PCR results showed *E. histolytica* only, suggesting elimination of *E. dispar* from culture by *E. histolytica*.

It was proved for the first time in Poland that a person (the wife of an infected man) who did not leave Poland herself was infected with *E. dispar* from the husband who previously travelled to different regions of the world. The identical nonpathogenic zymodeme (I) and the profiles of PCR products obtained with RAPD 3 primers (Fig. 1C) indicated the identity of these two isolates especially that the PCR profiles obtained for other *E. dispar* and *E. histolytica* isolates were different. Clark and Diamond (1993) performed similar investigations permitting the differentiation of *E. histolytica* isolates

It is also worth noting the occurrence of the invasive amoebiasis (ulcer of the intestine, liver abscess) in a patient (No. 340) who did not leave the country but worked on a ship repaired in the Gdańsk shipyard and eat meals prepared by a Liberian cook on that ship. The patient was admitted to our clinic following the previous resection of the intestine fragment (amoeba trophozoites were detected in histological preparations) and institution of antiamoebic treatment. Hence, the stool examination (including PCR) in that period gave a negative result while the serological reactions were highly positive. This is one of several known cases of invasive amoebiasis in Polish citizens who did not leave the country (Jaroszewicz 1932, Rybicka-Stryjecka and Perlińska-Schneider 1967, Łachecki 1972). Twelve persons from the patient's family and co-workers from shipyard were also examined but in none of the cases E. *histolytica* infection was detected.

The examinations carried out demonstrated (Table 1) that in Poland, among the imported and indigenous strains of *E. histolytica sensu lato*, *E. dispar* species occurred most frequently (90 %), the phenotype of which, i.e. zymodeme I, was the most common one. *E. histolytica* occurred more rarely (15%), but also in persons who did not travel abroad. The results of isoenzymatic examinations, except one, were in good accordance with the PCR and serological tests. In that latter method, all but two sera from persons infected with *E. dispar* were serologically negative, and sera of 5 out of 6 persons infected with *E. histolytica* were positive in several tests.

Comparing our results with those reported by German authors (Walderich *et al.* 1997), a conclusion can be

drawn that the ratio of *E. histolytica* to *E. dispar* infections was almost 3-fold lower in Poles than in Germans returning from areas endemic for amoebiasis. One possible explanation may be the time spent in the tropics. According to Walderich *et al.* (1997), the Germans staying in the tropics for a short time, not exceeding 3 months, were more frequently infected with *E. histolytica* than those stayed there for a longer time, while the Poles usually stayed in the tropics much longer, for several years. Also the incidence of *E. histolytica sensu lato* infection was lower in Poles (0.63% according microscopic examinations) than in Germans and foreigners coming from those regions. The reasons can be sought for in the choice of the study group. In Poland, all persons reporting after the return to the country are examined, irrespective of clinical symptoms. This is due to the control examinations, including parasitological ones obligatory, after the official stay in tropical countries. Moreover, the group of 0.63% of the Poles infected with *E. histolytica sensu lato* comprises persons who returned to the country, while in examinations of Walderich *et al.* (1997), 5.1% of infected cases pertained in 2/3 to Germans and in 1/3 to foreigners visiting Germany.

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

**Elda GAINO and Manuela REBORA**

**Dipartimento di Biologia Animale ed Ecologia, Perugia, Italy**

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

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

## INTRODUCTION

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

Address for correspondence: Elda Gaino, Dipartimento di Biologia Animale ed Ecologia, Via Elce di Sotto, 06123 Perugia, Italy; Fax: ++39755855733; E-mail: gaino@unipg.it

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

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

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

## MATERIALS AND METHODS

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

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

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

## RESULTS

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

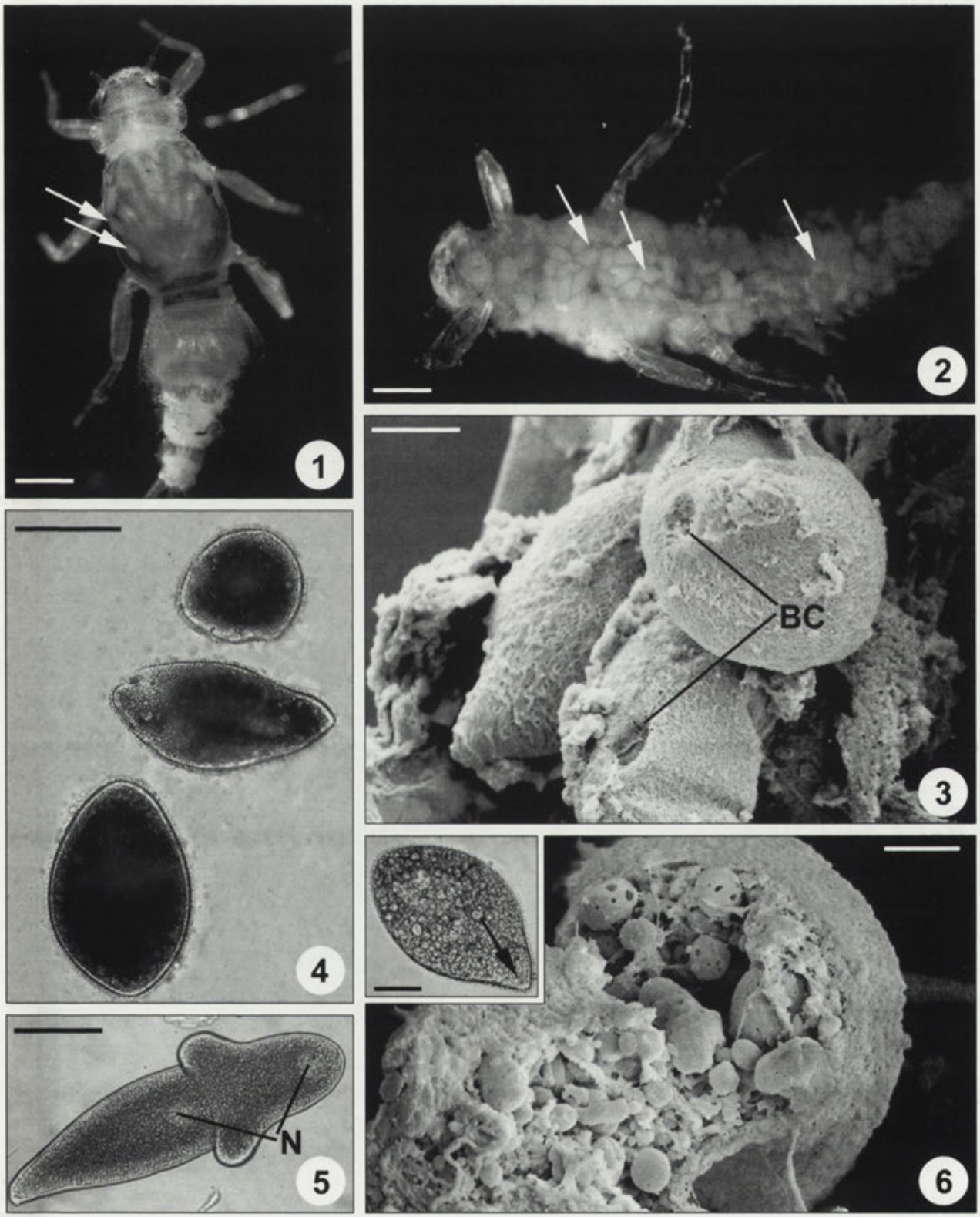
After their removal from the hemolymph, about one hundred ciliates were cultured in Petri dishes using the same stream water where infected specimens of *C. luctuosa* had been collected. In this medium, ciliates were freely moving and showed a different appearance, which probably reflected different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

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

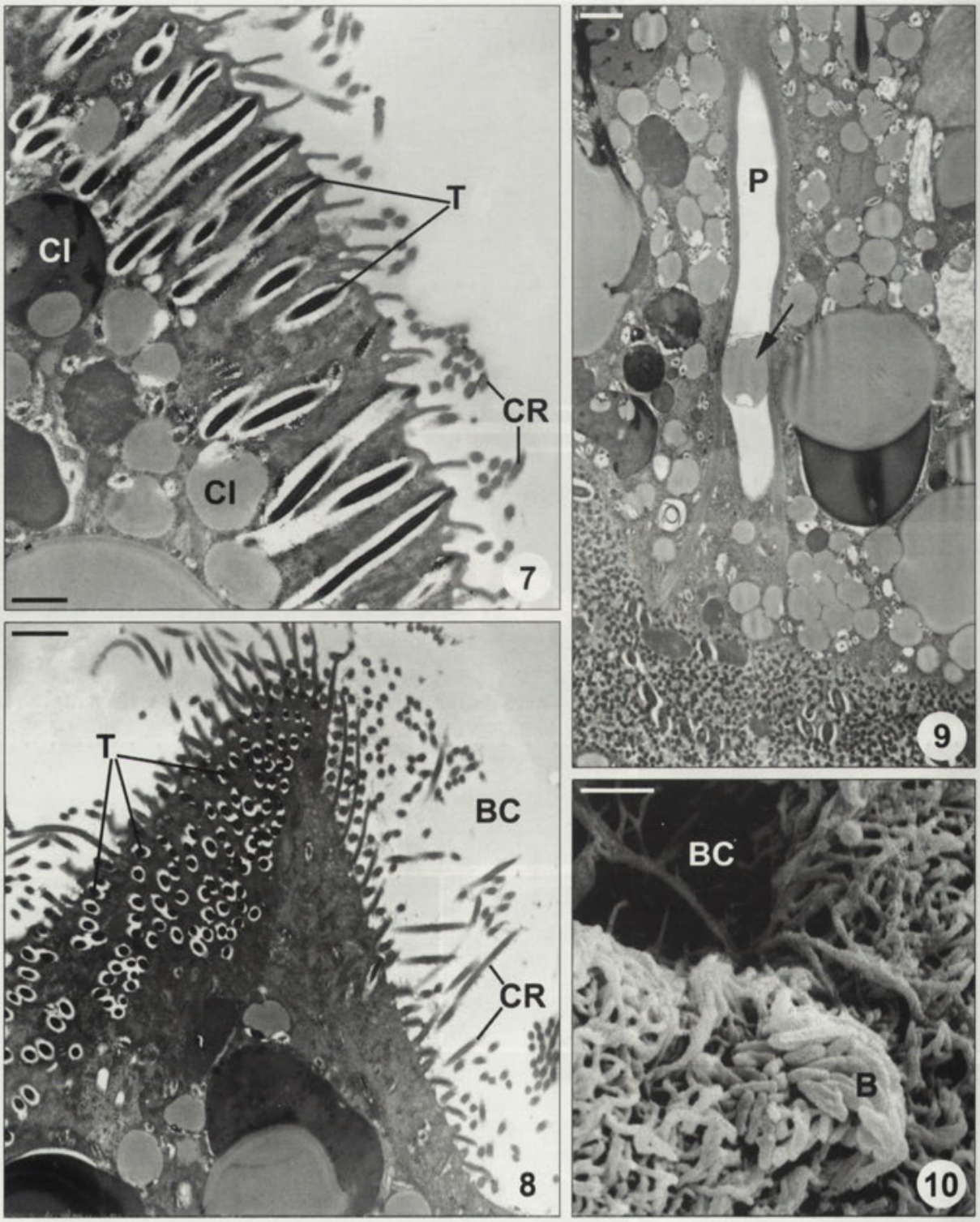
Two hours after removal from the host hemolymph, most of the parasites (about 60%) lost mobility and formed cysts. Twenty-four hours after the beginning of our experiments, bacterial attack affected both cysts and uncysted ciliates causing death of all the reared organisms.

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

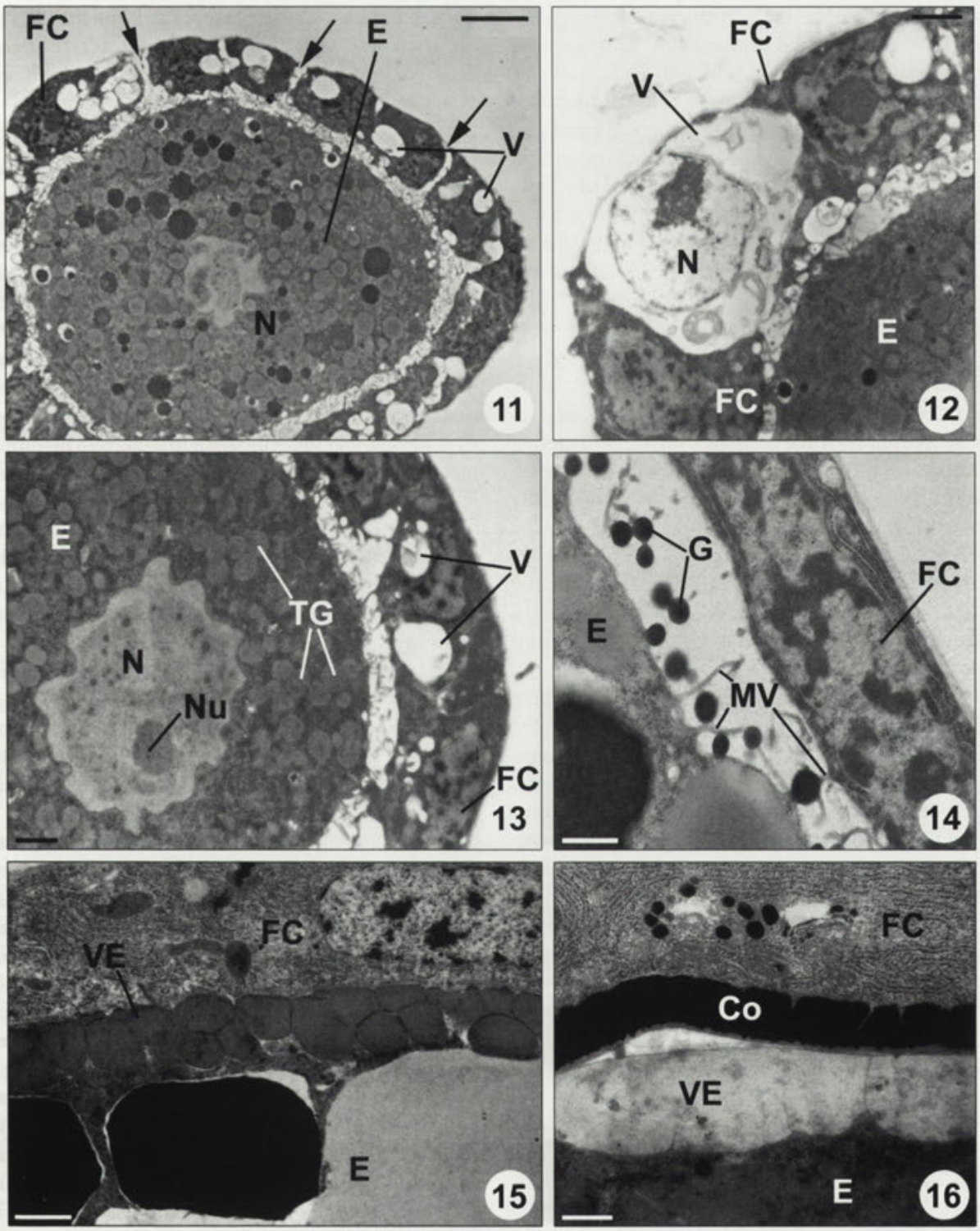
Among the freely moving *Ophryoglena* sp. removed from the hemolymph, trophonts were easily recognisable because of their spatulate apical region (inset of Fig. 6). Their cytoplasm was filled with inclusions of various sizes located just beneath the cell surface, as proved by




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




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




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

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

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

## DISCUSSION

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

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

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

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

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

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

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

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

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# Life Histories of Three New Coccidian Parasites from Three Coleopteran Stored-grain Pests of India

**Chhaya (Mitra) GHOSH, Amalesh CHOUDHURY and Kamales K. MISRA**

**Department of Zoology, Calcutta University, Calcutta, India**

**Summary.** Life histories of three new coccidian parasites (Protozoa: Apicomplexa: Coccidia) obtained from the larvae of three different coleopteran stored-grain pests, *Tribolium castaneum* Herbst, *Alphitobius piceus* Olivier and *Palorus ratzeburgii* Wissmann, respectively, have been described in detail. These coccidian parasites belong to the genus *Adelina* Hesse 1911 and are named as *Adelina castana* sp. n., *Adelina picei* sp. n. and *Adelina palori* sp. n. The complete life histories of these parasites are observed on the fat bodies along with the body fluid of the hosts. A comprehensive comparative account of three life histories is presented in tabular form.

**Key words:** Adeleidae, Apicomplexa, cereal pest, Coccidia, fat body, life history.

## INTRODUCTION

Hesse (1911) established the genus *Adelina* to accommodate the coccidian parasites, which differ from the members of the genus *Adelea* by the number and shape of the sporocyst in the oocyst. The sporocysts are fewer in number and are spherical instead of being discoidal. He transfered several species from the genus *Adelea* to *Adelina* such as *A. octospora* (H), *A. simplex* (Schneider, 1885), *A. tipulae* (Leger, 1897) *A. mesnili* (Perez, 1903), *A. akidini* (Leger, 1900), *A. transita* (Leger, 1904) and *A. zonula* (Moroff, 1906). Afterwards several species were added to this genus, one *Adelina* sp. by Chatton (1912), *A. domidiata* (Schneider, 1885) by Schellack (1913), *A. tenebrionis* by Sautet (1930), *A. tribolii* by Bhatia (1937), *A. cryptocerci* by Yarwood (1937), *A. schellacki* by Ray and Dasgupta (1937), *A. doronis* by Hauschka (1943), *A. sericesthis* by Weiser and Beard (1959), *A. melolonthae* by Tuzet *et al.* (1965), *Adelina* sp. by Hall *et al.* (1971), *A. riouxi* by Rioux *et al.* (1972) and *A. collembolae* by Purrini (1984). This article presents a comparative study of the morphology and life histories of three new coccidian parasites, *Adelina castana* sp. n., *A. picei* sp. n. and *A. palori* sp. n. obtained from the fat body and body fluid of three different coleopteran host insects *Tribolium castaneum* Herbst, *Alphitobius piceus* Olivier and *Palorus ratzeburgii* Wissmann belonging to the family Tenebrionidae.

## MATERIALS AND METHODS

Both the larvae and adults of the 3 coleopteran cereal pests *Tribolium castaneum* Herbst, *Alphitobius piceus* Olivier and *Palorus ratzeburgii* Wissmann of the family Tenebrionidae were collected from

Address for correspondence: Kamales K. Misra, 156/84 Banerjee Para Road, Behala, Calcutta 700 060, India; E-mail: misrakk@vsnl.com

different cereal samples of warehouses and granaries in and around Calcutta (West Bengal), India. The smears of body fluid and fat body of the larvae of the pests were prepared with a drop of 0.5% saline water. The smears were fixed with normal and warm (40-50°C) Schaudinn's fluid (Chen 1948) and stained in iron-alum-haematoxylin. Saturated picric acid was also used for more effective clear differentiation (Tuan, 1930). Measurements were made using a calibrated micrometer eyepiece. Parasites were transmitted to new hosts by feeding spores in the food. Camera lucida drawings were made and photomicrographs were taken by a Leica M4-2 camera.

## OBSERVATIONS

### *Adelina castana* sp. n.

Merogony: the sporozoites are small, slender vermicular, one end of which tapers and the other end is blunt. The nucleus is a compact spherical body and centrally located (Figs. 1A, 2). The matured sporozoites in the body fluid are more or less sausage-shaped mobile body. In the fat body or connective tissue, it becomes stationary and grows to an oval or spherical schizont (meront) (Figs. 2, 3). The nuclear membrane is not visible under light microscope. The nucleus breaks into several small nuclei. The cytoplasm splits and is arranged around each of these nuclei and becomes an elongated merozoite. The schizont is now a bunch of merozoites, closely apposed to each other along their longitudinal axis (Figs. 1B, 4). The number of merozoites in each schizont varies from 4 - 20. The free merozoite is an elongated sickle-shaped body, with one end is more pointed than the other and a round nucleus at the centre.

Gamogony: macrogametocytes are stationary and are more or less oval in shape (Figs. 1D, 6), where as the microgametocytes are small and usually round (Figs. 1E, 6). Subsequently, the microgametocyte adheres to macrogametocyte and becomes partly embedded in the body of it (Fig. 7). In some cases, more than one microgametocyte is found attached with the macrogametocyte.

Fertilization: macro- and microgametes are formed from the respective gametocytes. One of the microgametes fertilizes the macrogamete and forms a zygote. A cyst wall is formed around it and is termed as oocyst (Fig. 8). The remains of the microgametocyte are seen attached to the zygote wall for some time.

Sporogony: after fertilization the nucleus of the zygote becomes more or less spindle-shaped. The spindle simply is stretched and broken in an irregular manner giving rise to several masses of chromatin materials. The cytoplasm of the zygote divides by a process of budding into sporoblasts with a nucleus at the centre. Several spherical sporoblasts are formed and are transformed into sporocysts and are covered by cyst wall, the diameter of which measures 8.5 µm in most cases. The sporocyst contains some residual bodies in addition to two sporozoites (Figs. 1E, 8, 9). The number of sporocysts in an oocyst varies from 4 to 12 while numbers 8 and 12 are common. Detail measurements are presented in the Table 1.

Material: containing life stages of the holotype no. Pt 2334 specimen is deposited in the National Zoological Collection, Zoological Survey of India, Calcutta and the slides number 1-6 C(1) T(5) containing several paratypes are deposited in the Parasitology Laboratory, Department of Zoology, University of Calcutta, Calcutta, India.

Site of infection: fat bodies, connective tissue and body fluid.

Type host: *Tribolium castaneum* Herbst

Type locality: Calcutta (West Bengal), India.

Etymology: the species name of this parasite is given after the species name of the insect host.

### *Adelina picei* sp. n.

Merogony: the sporozoites are small and slender vermicular. One end is narrow and the other end is blunt and wide. The nucleus is a compact spherical mass and centrally located (Figs. 10A, 11). The sporozoite develops into oval shaped schizont (Fig. 12). Nucleus of the fully formed schizont divides into several small nuclei. Each bit of the nuclei by accumulating cytoplasm, transforms into an elongated sickle-shaped merozoite. The number of merozoites in each schizont varies from 12 - 20 and they are usually closed to each other along their longitudinal axis (Figs. 10B, 13). The free merozoites either repeat the cycle or develop into gametocytes.

Gamogony: the macrogametocytes are large and more or less oval in shape where as the microgametocytes (Figs. 10C, D, 14), are small and round. The association between the micro- and macrogametocytes occurs after a period of growth of the gametocytes (Fig. 15) but not at the early stage of development like other species of *Adelina*. The nucleus of the macrogametocyte is a compact spherical body and is situated near the attachment of the microgametocytes (Fig. 17) unlike the other species of *Adelina*, where it is situated at opposite pole of the attachment of microgametocyte.

Fertilization: after fertilization the zygote is formed and a cyst wall envelops the macrogametocyte and an oocyst is formed. The remains of the microgametocyte are seen

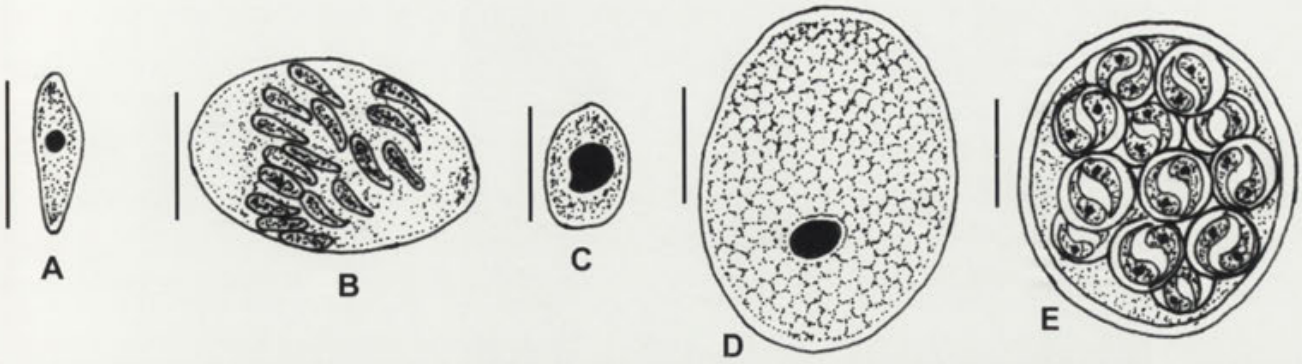
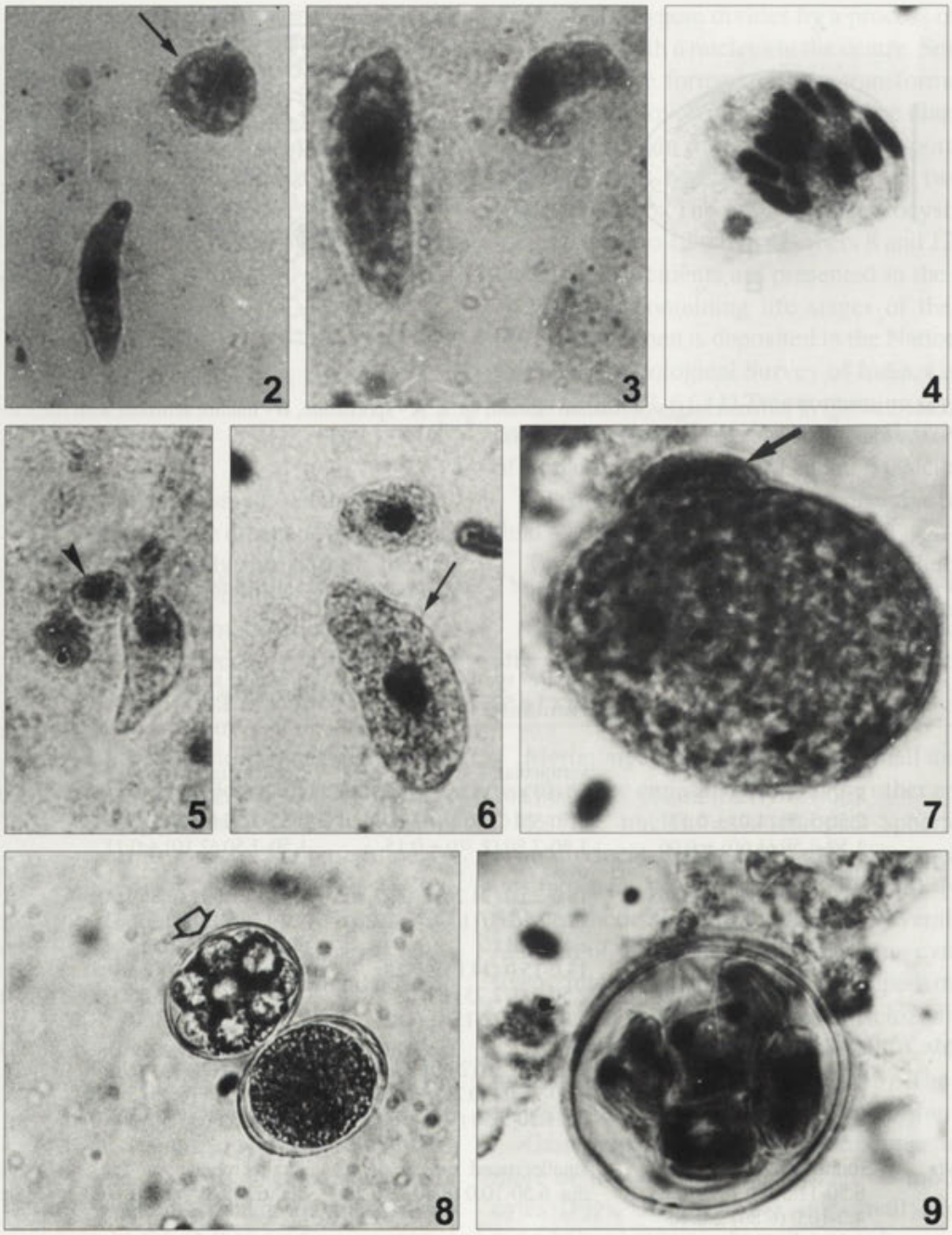



**Fig. 1.** Camera lucida drawings of different stages of *Adelina castana* sp. n. **A** - sporozoite, **B** - mature schizont with fully formed merozoites, **C** - microgametocyte, **D** - macrogametocyte, **E** - sporocysts in oocyst. Scale bars - 10μm

**Table 1.** Details of measurement (in μm) of different stages of 3 new species of coccidia, *Adelina castana* sp. n., *A. picei* sp. n. and *A. palori* sp. n. compared with *A. tribolii* Bhatia

| | *A. castana* sp. n.<br>R, $\bar{x}$ [1] ± S.E. | *A. picei* sp. n.<br>R, $\bar{x}$ [2] ± S.E. | *A. palori* sp. n.<br>R, $\bar{x}$ [2] ± S.E. | *A. tribolii* Bhatia |
|---|---|---|---|---|
| **Sporozoite** | slender vermicular | vermicular | slender slightly curved | small slender |
| L | 8.50-13.0 (11.35) ± 0.37 | 10.0-13.0 (11.95) ± 0.30 | 11.50-16.50 (13.45) ± 0.57 | 9.0 x 1.2-2.0 |
| W | 2.50-5.50 (4.0) ± 0.17 | 2.50-3.0 (2.80) ± 0.07 | 2.50-3.50 (2.65) ± 0.10 | - |
| N | 1.50-2.50 (1.60) ± 0.06 | 1.50-2.50 (2.10) ± 0.15 | 1.50-2.50 (2.10) ± 0.11 | - |
| **Meront** | oval or spherical | oval | oval | oval or spherical |
| L | 18.0-30.0 (22.75) ± 0.63 | 18.0-25.0 (21.20) ± 0.57 | 16.50-21.50 (18.85) ± 0.50 | 13.5-30.0 |
| W | 13.0-18.0 (14.90) ± 0.38 | 11.50-16.50 (14.45) ± 0.28 | 8.0-15.0 (11.15) ± 0.63 | 6.25-20.0 |
| **Merozoite** | elongated sickle shaped | elongated | elongated sickle shaped | small vermicular |
| L | 8.50-13.0 (11.65) ± 0.32 | 13.0-15.0 (14.0) ± 0.28 | 12.50-18.0 (15.90) ± 0.51 | 15.0 |
| W | 1.50-3.50 (2.60) ± 0.10 | 2.50-3.0 (2.75) ± 0.08 | 2.50-5.0 (4.15) ± 0.26 | 3.0 |
| N | 1.50-3.50 (2.60) ± 0.10 | 1.50-2.50 (2.15) ± 0.12 | 2.0-3.0 (2.75) ± 0.10 | - |
| **Macrogametocyte** | oval | oval | oval | oval |
| L | 13.0-33.0 (24.30) ± 1.27 | 21.50-31.50 (28.0) ± 1.09 | 15.0-30.0 (20.15) ± 1.29 | 20.8-48.6 |
| W | 6.50-30.0 (18.97) ± 1.54 | 13.0-25.0 (20.25) ± 1.18 | 10.0-21.50 (16.35) ± 1.05 | 15.6-32.5 |
| LN | 2.50-10.0 (5.15) ± 0.47 | dia 1.50-5.50 (3.8) ± 0.43 | dia 3.0-3.50 (3.30) ± 0.07 | - |
| WN | 2.50-6.50 (4.30) ± 0.25 | - | - | - |
| **Microgametocyte** | round or oval | smaller round | smaller round | rounded |
| L | 8.50-11.50 (9.70) ± 0.23 | dia 6.50-10.0 (8.40) ± 0.29 | dia 6.0-8.0 (7.30) ± 0.23 | dia 7.8-15.0 |
| W | 4.0-10.0 (6.80) ± 0.40 | - | - | - |
| N (Dia) | 1.50-3.50 (2.50) ± 0.16 | 2.50-3.0 (2.65) ± 0.07 | 2.0-2.50 (2.30) ± 0.07 | - |
| **Oocyst** | large oval | more or less spherical | oval | oval or spherical |
| L | 20.0-33.0 (29.32) ± 0.73 | 28.0-38.0 (33.95) ± 1.07 | 16.50-41.0 (30.30) ± 2.09 | 26.0-50.0 |
| W | 15.0-30.0 (25.45) ± 0.67 | 25.0-35.0 (29.95) ± 0.90 | 13.00-33.0 (24.60) ± 1.63 | 22.5-36.5 |
| **Sporocyst** | round | round | round | round |
| Dia | 6.50-10.0 (8.20) ± 0.21 | 8.0-10.0 (8.50) ± 0.25 | 8.0 | 10.4 |
| No. of sporocyst in oocyst | 4 -12 (8, 12) | 8 - 18 (12) | 4 -12 (8) | 2 - 14 (8, 12) |
| **Type host** | *Tribolium castaneum* Herbst | *Alphitobius piceus* Ol | *Palorus ratzeburgii* Wissmann | *T. ferrigineggum* F. |
| **Locality** | Calcutta, India | Calcutta, India | Calcutta, India | Cambridge, U.K. |

Abbreviation used: R - range; $\bar{x}$ - mean; S.E. - standard error; L - length; LN - length of the nucleus; N - nucleus; W - width; WN - width of the nucleus; Dia - diameter; [1] - 20 samples; [2] - 30 samples




**Figs. 2-9**. Photomicrographs of different stages of *Adelina castana* sp. n. **2** - sporozoite with nucleus at the middle and an early meront (arrow), x 1300; **3** - early schizont, x 745; **4** - mature meront, merozoites are arranged in longitudinal axis, x 1056; **5** - merozoites are developing into male (arrowhead) and female gametocytes, x 1323; **6** - micro- and macrogametocytes (arrow), x 700; **7** - micro- (arrow) and macrogametocytes are in association, x 1500; **8** - early and late (arrow) oocysts (*in vivo*), x 635; **9** - enlarged oocyst showing sporozoites in sporocysts in stained preparation, x 1334

to attach with the oocyst for some time. The oocyst increases in size and becomes more or less spherical in shape.

Sporogony: the zygote nucleus divides into several small units. Each one of them becomes round and accumulates lump of cytoplasm around it and is known as sporoblast (Fig. 10E). Each sporoblast is covered by a wall and transforms into sporocyst. The round sporocyst contains two sporozoites. The number of sporocyst in an oocyst (Fig. 16) varies from 8 - 18, while 12 are common.

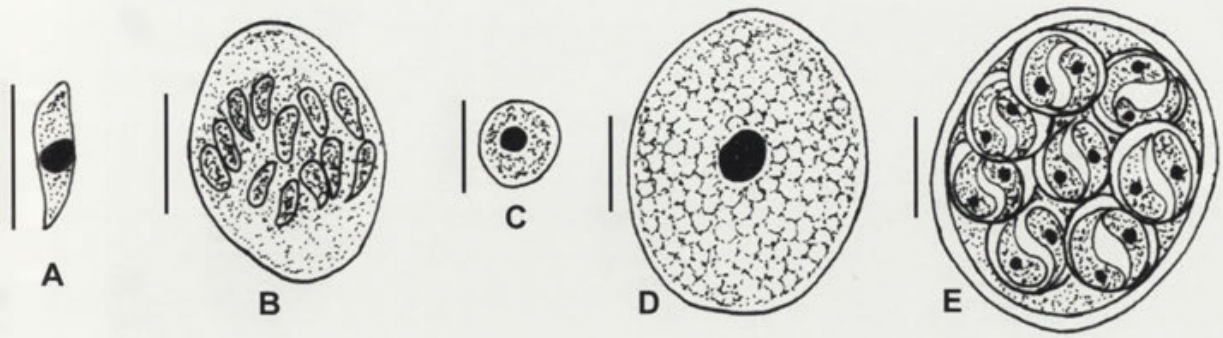



**Fig. 10**. Camera lucida drawings of different stages of *Adelina picei* sp. n. **A** - sporozoite; **B** - mature schizont with fully formed merozoites; **C** - microgametocyte; **D** - macrogametocyte; **E** - sporocysts in oocyst. Scale bars - 10μm

The measurements of all the stages are tabulated (Table 1).

Material: holotype no. Pt. 2335 containing the life stages of the new species is deposited in the National Zoological Collection of the Zoological Survey of India, Calcutta and the slides 9-10C(1)T(5) containing paratypes are deposited in the Parasitology Laboratory, Department of Zoology, University of Calcutta, Calcutta 700 019, India.

Infection site: fat body and body fluid.

Type host: *Alphitobius piceus* Olivier.

Locality: Calcutta (West Bengal), India.

Etymology: the species name of the coccidian parasite is coined after the species name of the host.

***Adelina palori* sp. n.**

Merogony: the sporozoites are small, slender curved vermicules. The one end is blunt and wide while the opposite end is tapering (Figs. 18A, 19). The sporozoite develops into more or less oval shaped meront (early schizont) (Fig. 20). The fully formed meront contains a cluster of elongated merozoites. The number of merozoites varies with the size of the meront, and ranges from 8 - 14; while 12 is mostly common (Figs. 18B, 21). The merozoites liberate by disintegration of the outer wall of the meront. The free merozoites either repeat the merogony or develop into gametocytes.

Gamogony: the microgametocyte is small and round with a distinct nucleus (Figs. 18C, 22). The macrogametocytes are large oval bodies with clear cytoplasm and prominent nucleus at one side (Figs. 18D, 22). The microgametocyte adheres to the macrogametocyte from the very early stage of development. After sometime, the microgametocyte becomes partly embedded in the body of the macrogametocyte (Fig. 23). Occasionally more than one microgametocyte is seen to attach to the macrogametocyte (Fig. 23).

Fertilization: after fertilization the zygote is enclosed in a double-walled cyst to transform into an oocyst. Remains of microgametocyte is found to attach to the zygote for sometime.

Sporogony: the nucleus of the zygote breaks into several chromatin granules. Each of the chromatin materials develops into sporoblast by accumulating cytoplasm around it. The spherical sporoblast is covered by a cyst wall and transforms into a round sporocyst (Figs. 18E, 24). The number of sporocyst in the oocyst varies from 4 - 12, while 8 are more common. Each sporocyst produces two sporozoites. Detail of the measurements is given in the Table 1.

Material: holotype no. Pt. 2336 containing life stages is deposited in the National Zoological Collection of the Zoological Survey of India, Calcutta and the slides 13-14C(1)T (5) containing paratypes are deposited in the Parasitology Laboratory, Department of Zoology, University of Calcutta, Calcutta 700 019, India.

Infection site: fat body and body fluid.

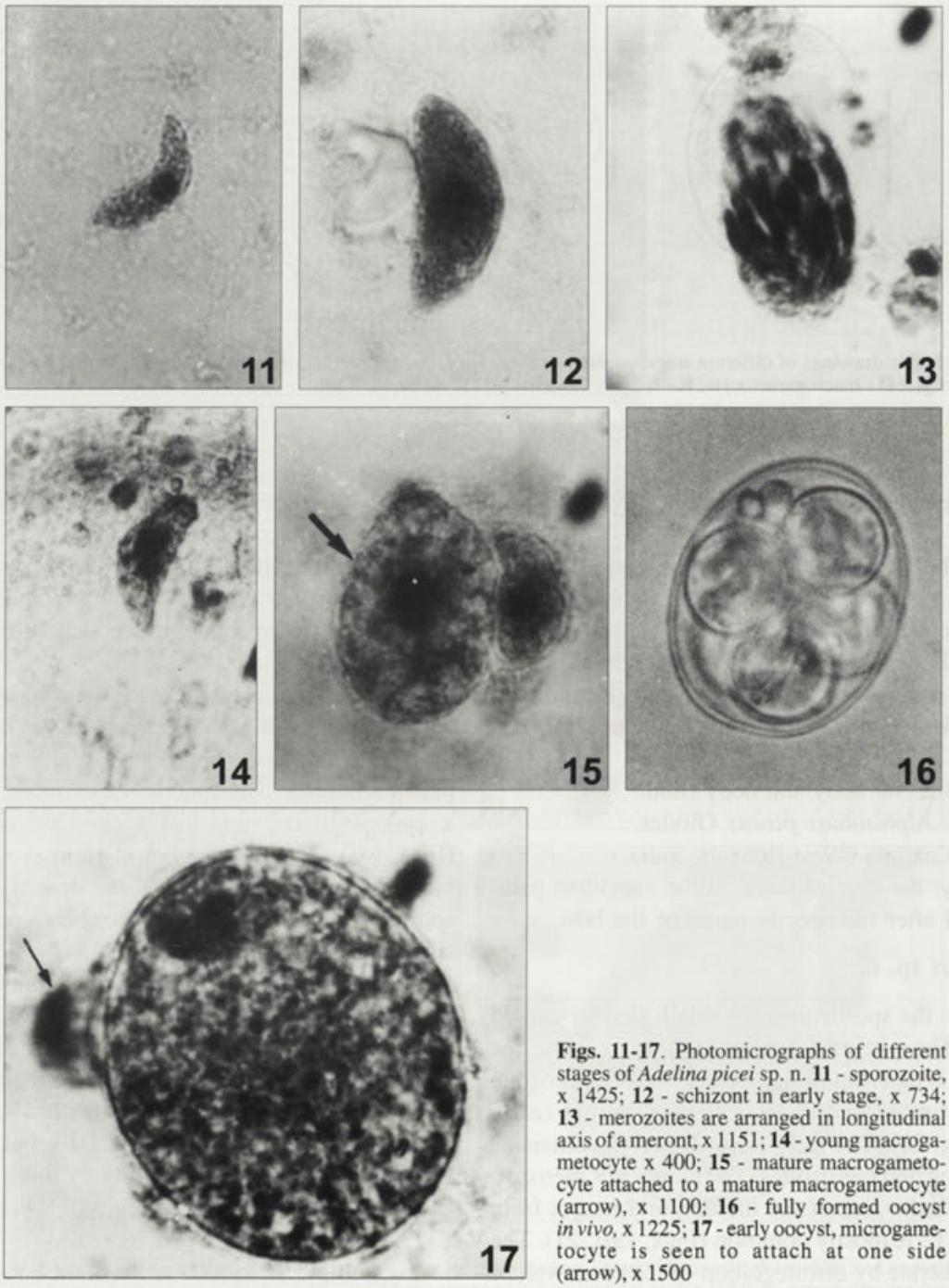
Type host: *Palorus ratzeburgii* (Wissmann).

Locality: Calcutta (West Bengal).

Etymology: the species name of the coccidian parasite is coined after the generic name of the coleopteran host.

## DISCUSSION

Coccidian parasites of the genus *Adelina* have spherical or subspherical oocyst; spherical thick-walled sporocysts are fewer in number and are parasites of arthropods and oligochaetes. Only two species of the genus *Adelina* have been described from tenebrionid beetles. One is




**Figs. 11-17**. Photomicrographs of different stages of *Adelina picei* sp. n. **11** - sporozoite, x 1425; **12** - schizont in early stage, x 734; **13** - merozoites are arranged in longitudinal axis of a meront, x 1151; **14** - young macrogametocyte x 400; **15** - mature macrogametocyte attached to a mature macrogametocyte (arrow), x 1100; **16** - fully formed oocyst *in vivo*, x 1225; **17** - early oocyst, microgametocyte is seen to attach at one side (arrow), x 1500

*Adelina tenebrionis* from *Tenebrio molitor* described by Soutet in 1930 (redescribed in details with the help of ultrastructure by Malone and Dhana in 1988) and the other one is *A. tribolii* from *Tribolium ferrugineum* by Bhatia in 1937. Both the species are, however, described from outside the Indian subcontinent (Riley and Krogh 1927).

The oocyst of *Adelina castana* sp. n., *A. picei* sp. n. and *A. palori* sp. n. are round to slightly oval in shape. The number of rounded thick-walled sporocysts is 4 - 12 in *A. castana*, 8 - 18 in *A. picei* and 8 on average in *A. palori*. These parasites inhabit in 3 different coleopteran hosts: *Tribolium castaneum* Herbst, *Alphitibious picius* Olivier and *Palorus ratzeburgii*

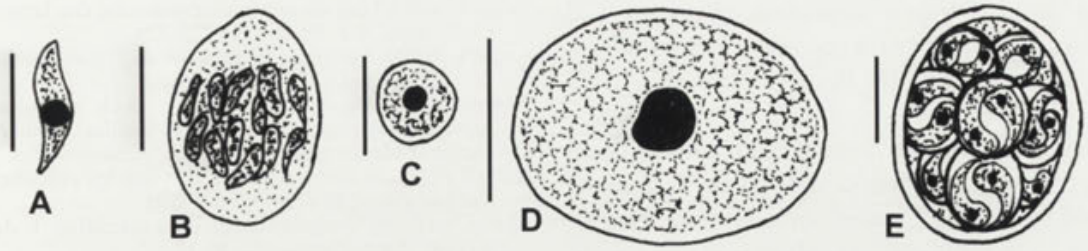
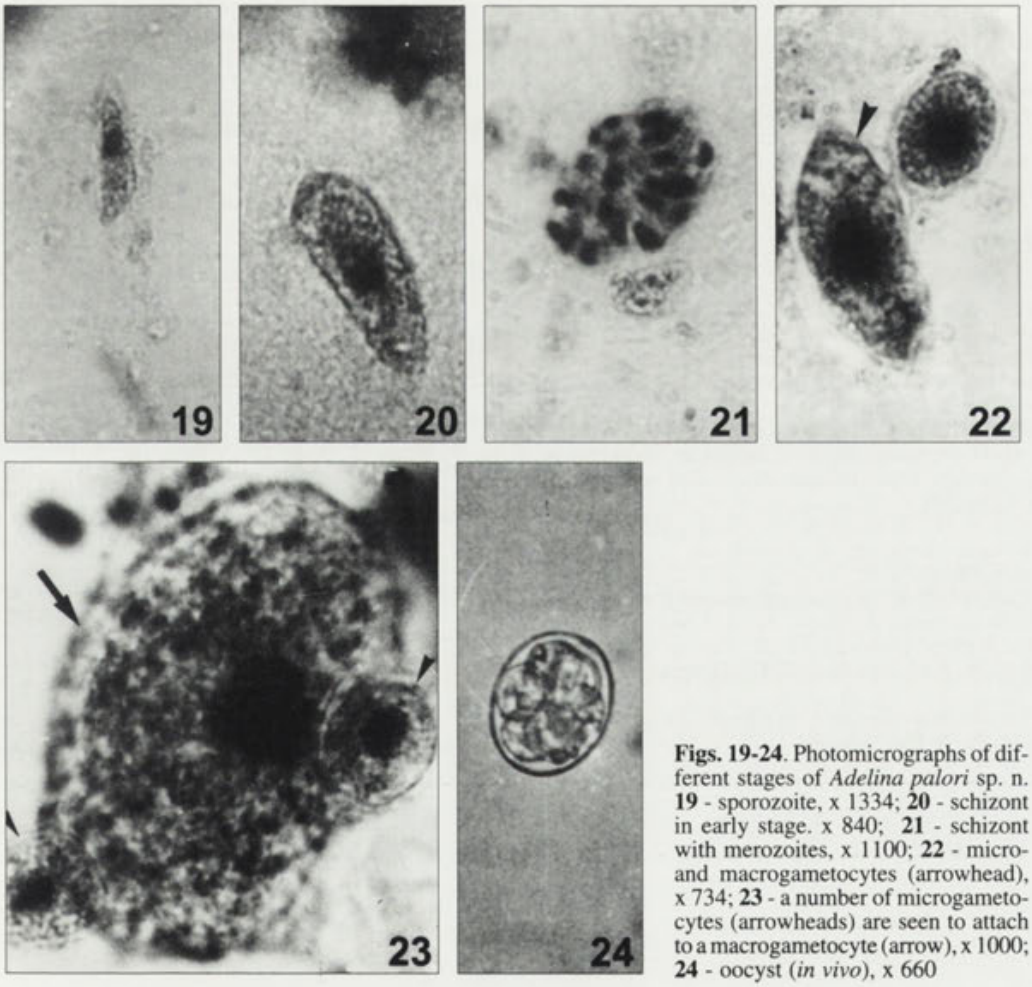



**Fig. 18**. Camera lucida drawings of different stages of *Adelina palori* sp. n. **A** - sporozoite; **B** - mature schizont with fully formed merozoites; **C** - microgametocyte; **D** - macrogametocyte; **E** - sporocyst in oocyst. Scale bars - 10μm




**Figs. 19-24**. Photomicrographs of different stages of *Adelina palori* sp. n. **19** - sporozoite, x 1334; **20** - schizont in early stage. x 840; **21** - schizont with merozoites, x 1100; **22** - micro- and macrogametocytes (arrowhead), x 734; **23** - a number of microgametocytes (arrowheads) are seen to attach to a macrogametocyte (arrow), x 1000; **24** - oocyst (*in vivo*), x 660

Wissmam of family Tenebrionidae respectively. So, these coccidian parasites under communication may safely be placed in the genus *Adelina* under the family Adeleidae Mesnil 1903 (Wenyon, 1926). Mensural detail of all the 3 new species and *A. tribolii* Bhatia is compared in Table 1. The insufficient description of *A. tenebrionis* reveals that the diameter of the spherical oocyst is 20 - 30 μm, but it is more or less oval in the coccidian species under description. The sporocysts are 2 to 12 in *A. tenebrionis* but it is 4 - 12 in *A. castana*, 8 - 18 in *A. picei* and 8 on average in *A. palori* (Table 1). A meticulous comparison of the morphometrics

among the proposed new coccidian parasites and *A. tribolii* Bhatia reveals that the coccidian members from different hosts differ in detail mensural data (Table 1).

With reference to the above the present authors deem it necessary to attribute these coccidian parasites from the 3 tenebrionid insect hosts: *Tribolium castaneum* Herbst, *Alphitibious picius* Olivier and *Palorus ratzeburgii* Wissmann, as separate identity with new species status and propose to coin them as *Adelina castana* sp. n., *Adelina picei* sp. n. and *Adelina palori* sp. n. respectively.

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ACTA
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# *Becnelia sigarae* gen. n., sp. n. Isolated from Testes of the Water Boatmen, *Sigara lateralis* (Heteroptera: Corixidae) in the Czech Republic

**Tomáš TONKA and Jaroslav WEISER**

**Institute of Entomology, ASCR, České Budějovice, Czech Republic**

**Summary.** A microsporidian *Becnelia sigarae* gen. n., sp. n. (Microspora: Amblyosporidae) was isolated from testes of a water boatman *Sigara lateralis* (Heteroptera: Corixidae) near Bavorov, South Bohemia, Czech Republic. The life cycle of the pathogen includes a merogony with uninucleate stages, a meiotic sequence in part with binucleate stages (diplokarya) and a sporogony resulting in a persistent sporophorous vesicle containing eight spores. Spores are long oval, slightly curved, with broader basis and equally rounded ends, 5 ± 0.5 x 2.5 ± 0.5 μm in diameter. A series ending with early spores serving for autoinfection in the primary host and spread of the infection in other tissues differs in ultrastructure of spores. They are shorter and more constricted apically and measure 4 x 2.5 ± 3 μm. Both spore types have a polaroplast with a central part with multiple broad chambers enclosed anteriorly and posteriorly in circular layers of dense lamellae. The spore wall of both types is characterized by a thin exospore and an endospore of equal thickness all over the spore with exception of the attenuated apical pole. Mature spores have an anisofilar polar filament coiled in 9 - 11 turns with first 5 - 6 broader turns and 4 - 5 narrower turns. The early spores have the filament coiled in 7 turns, of which 4 are broader and 3 narrower. All characteristics of the new microsporidium reveal that it is similar to different *Amblyosporidae* and we therefore propose to include it into this family. The new genus *Becnelia* is proposed with *B. sigarae* as a type species. The taxonomic position as well as the relationships to other microsporidia described from Heteroptera are discussed.

**Key words**: *Becnelia sigarae* gen. n., sp. n., Heteroptera, life cycle, microsporidia, *Sigara*, taxonomy, ultrastructure.

**Abbreviations:** a - anchoring disc, D - disporous sporogony, e - exospore, E - endospore, er - endoplasmic reticulum, F - polar filament ($F_1$ - narrow part), g - gamet (planont), G - Golgi system, h - hinge, m - system of multiple membranes, M - meront, Me - meiont, n - nucleolus, N - nucleus, o - granular secretions, O - early spores, p - plasmalemma, P - lamellar polaroplast, $P_1$ - broad chamber polaroplast, Ps - polar sac, r - polysomes, R - rosette stage, S - mature spores, Sb - sporoblast, sc - synaptonematic complex, Sn - sporonts, Sp - sporogonial plasmodia, Sw - wrinkled sporoblast, t - tubular secretions, u - umbrella, v - sporophorous vesicle,V - system of vacuoles.

## INTRODUCTION

From different Heteroptera at least 9 microsporidian species were described up-to-date, mainly from the aquatic bugs (Sprague 1977). None of them has been studied using electron microscopy, as far as we know and there are no data on their ultrastructures and a complete life cycle.

One infected adult water boatman, *Sigara lateralis* (Leach, 1817) (Heteroptera: Corixidae) was found in a large population collected in a temporary pool. The infected male had an orange coloured abdomen in the

Address for correspondence: Tomáš Tonka, Department of Insect Pathology, Institute of Entomology, ASCR, Branišovská 31, CZ-370 05 České Budějovice, Czech Republic; Fax: +420 38 5300354; E-mail: tonka@entu.cas.cz

region of gonads and was dissected for identification of the parasite. A microsporidian with octosporous sporophorous vesicles was found in the testes of the male animal. *Toxoglugea gerridis* Poisson, 1941, *T. mercieri* (Poisson, 1924) Jírovec, 1936 both with horseshoe - like bent spores, *Thelohania veliae* Poisson 1928 with large oval spores, *Chapmanium nepae* (Lipa, 1966) Hazard and Oldacre, 1975 with navicular pansporoblasts and *Octosporea carlochagasi* Kramer, 1972 with tubular spores were other previously described octosporous species (Poisson, 1928; Jírovec, 1936; Hazard and Oldacre, 1975; Kramer, 1972). The first four were recorded from Heteroptera from Europe.

In this study we describe morphological features and the life cycle of a new microsporidian with octosporous sporophorous vesicle which differs from these mentioned above.

## MATERIALS AND METHODS

The infected adult male water boatman *Sigara lateralis* was collected in a temporary pool near the village Bavorov, NW from České Budějovice, South Bohemia, Czech Republic, in September 1998. A large group of water boatmen was brought to the laboratory and checked for presence of microsporidia. One single male had an orange coloured abdomen due to a microsporidian infection. It was dissected and from infected tissues smears were prepared, fixed in methanol and stained with 10% (v/v) Giemsa solution for 20 min. for inspection under the light microscope.

For electron microscopy, pieces of infected tissue were fixed in Karnovsky (2.5% glutaraldehyde, 2% paraformaldehyde) overnight at 4°C. After several washes in cacodylate buffer (pH 7.4) they were postfixed with 2 % osmium tetroxide for 2 h. Pieces of tissue were dehydrated in a graded aceton series and embedded in Durcupan. Semithin sections were stained with toluidin blue while ultrathin sections were stained with uranyl acetate and lead citrate in routine process and examined with a JEOL 1010 TEM at 80kV.

## RESULTS

### Pathology in the host

In the inspected group of water boatmen one single adult male was infected, with the infection located in the testes. The abdomen of the infected animal was coloured orange due to staining of the peritoneal sheat contrasted by spore masses. The epithelial cover and the germarium were the seat of the infection in the testes and the developmental stages of the microsporidian were found in the mass of sperm cells. The infected gonad was hypertrophic, filled with developmental stages of the pathogen. In the sample of water boatmen there was no further infected adult, male or female.

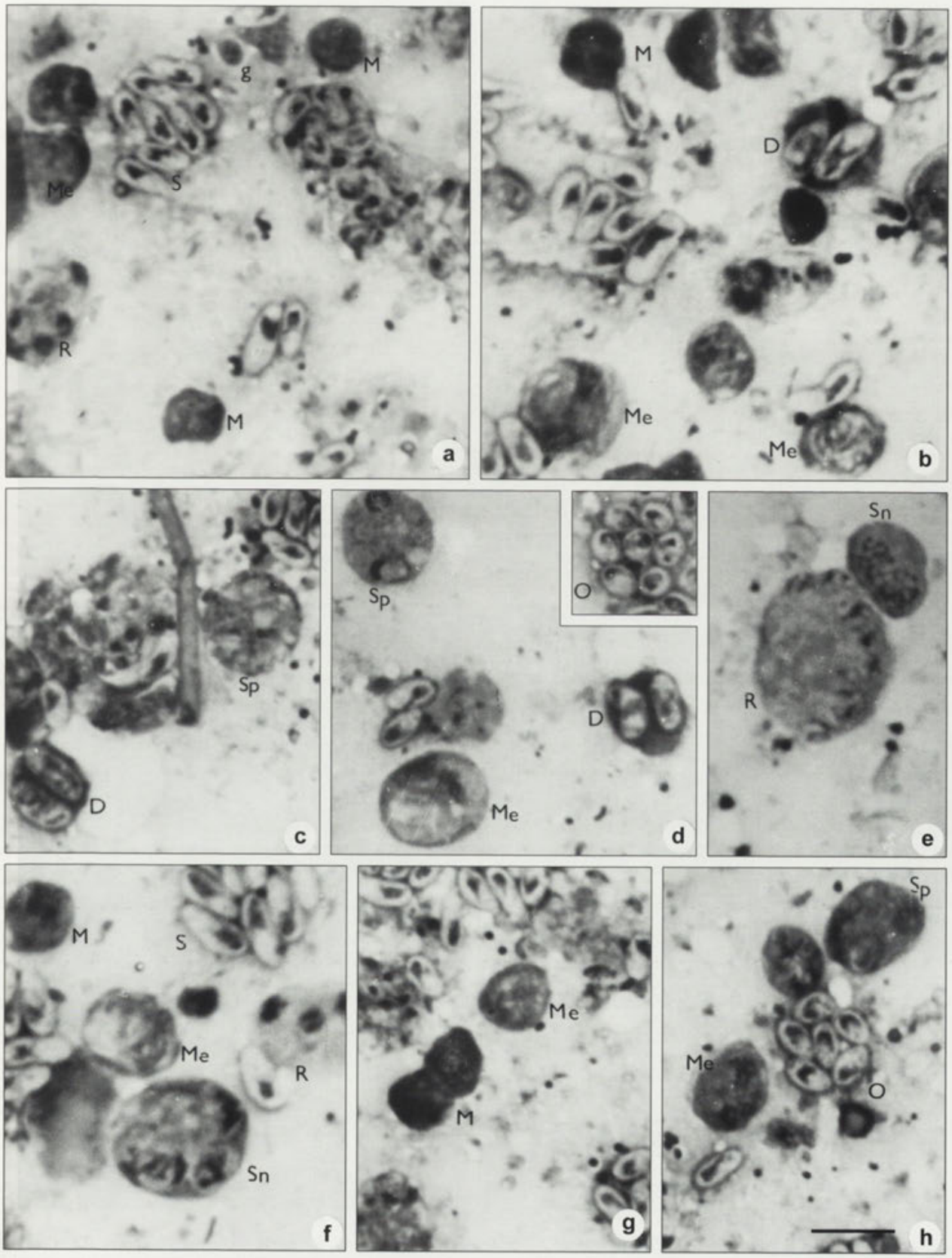
### Light microscopy

On smears different vegetative stages were present together with mature spores in round sporophorous vesicles. The development was divided in four principal phases: the early planont - gamet stage, the merogonial cycle, the cycle of meiotic division, the sporogony and spore maturation (Figs. 1, 15). The distribution of individual phases was evaluated among 2000 counted stages in Giemsa stained smears. Early stages identified as gametes (Figs. 1a-g) were small round cells 2 - 3 μm in diameter with dense cytoplasm and minute single round nucleus 1 - 2 μm in diameter. These stages were rare or difficult to identify, in the analysed sample were just 0.4% of the group. Meronts as next step in development were usually presented as deeply stained oval or irregular bodies (M in Fig. 1) with badly differentiated single nuclei and usually adhering in the smear to some sporophorous vesicles. They represented 2.8% of the evaluated group. Stages of meiotic division were the second series in the merogonial phase. They were represented by stages (Fig. 1 - Me) with a less stained cytoplasm and large nucleus. The size of these stages varied from 6 - 10 μm in diameter, usually the nucleus looked „empty", with chromosomes in some phase of division. Nuclei were 3 - 6 μm in diameter and the series ends with binucleate stages where the two nuclei adhered to each other as diplokarya. This stage represented 3.4% of the total. It ended with binucleate stages representing the first step in sporogony.

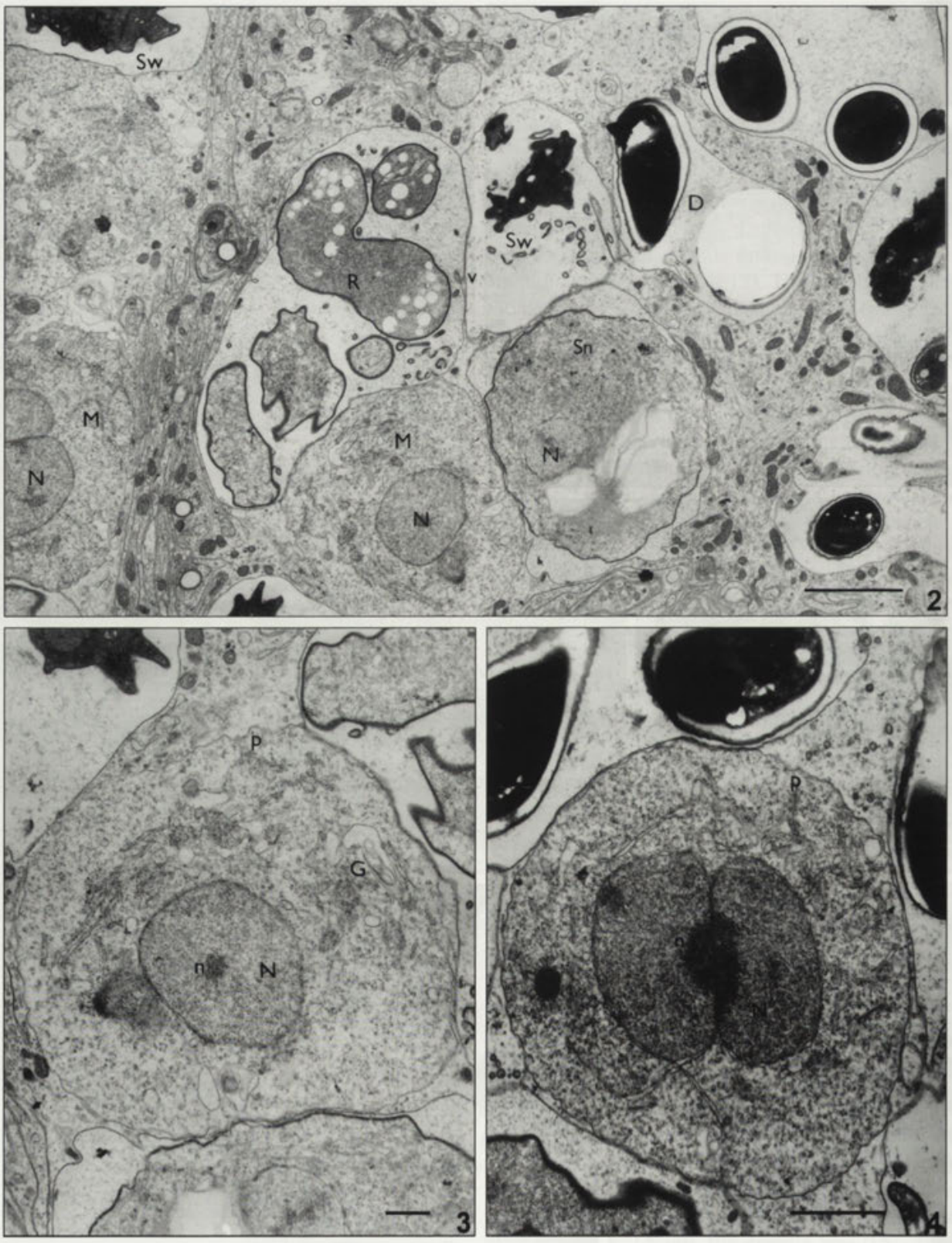
The next series, octonucleate plasmodia were produced in two subsequent nuclear divisions (1.5 %) (Fig. 1 - Sp). First the nuclei were distributed in the whole space of the stage, later (Fig. 1e - R) the nuclei were protruding on the border of the plasmodia and produced uninucleate budds which formed sporoblasts (rosettes, 1.2%). This stage was usually oval, 10 x 15 μm in diameter.

The octospores were enclosed in a persistent thin sporophorous vesicle. The octospore stage with prominent metachromatic red granules in the posterior pole indicating the position of the posterosome was a specific stage in spore maturation (15%). These young spores were oval, 5 x 2.5 μm, the granule was 0.5 - 1 μm in diameter.

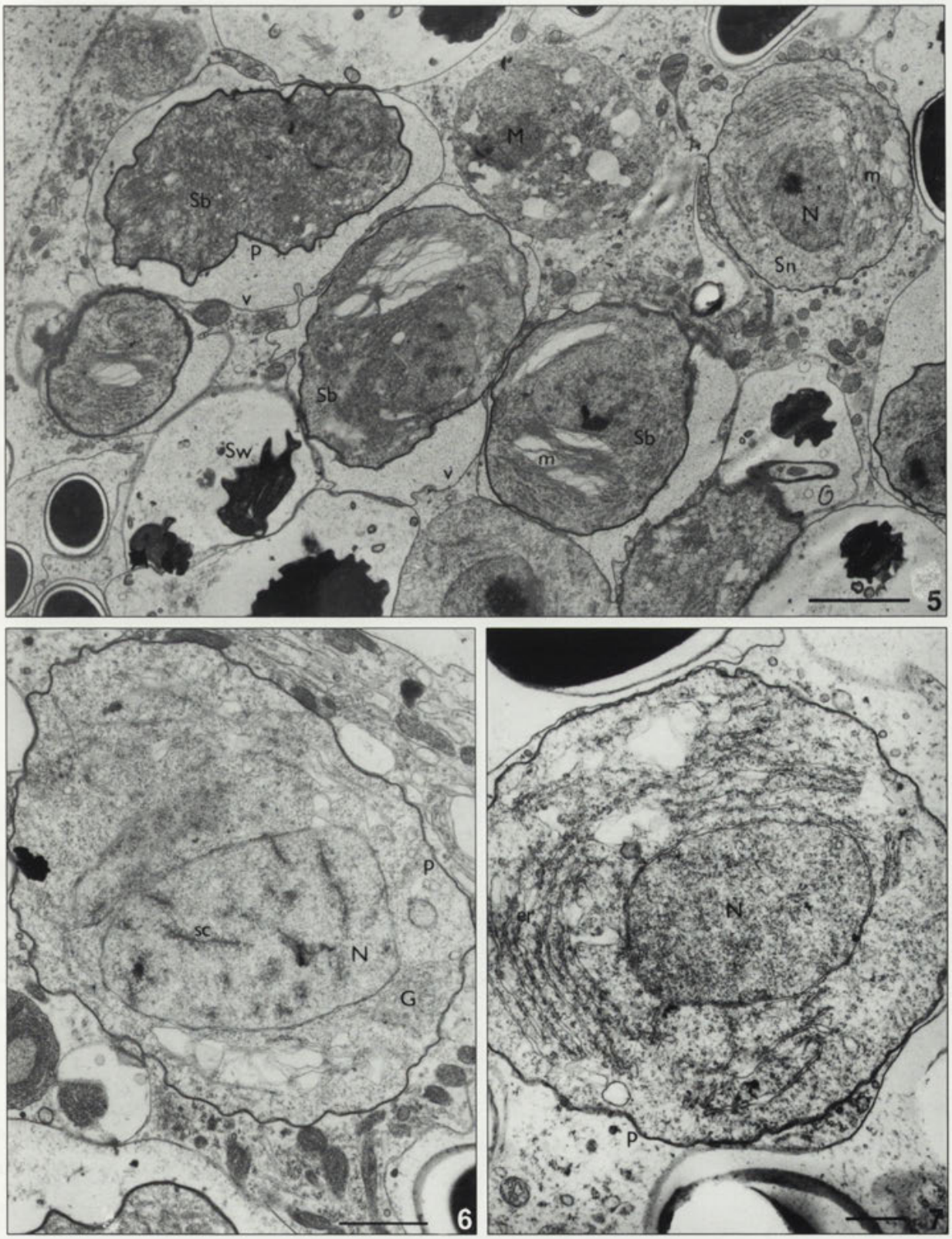
A sporophorous vesicle, 10-12 μm in diameter containing 8 slightly curved oval spores with broader basis and



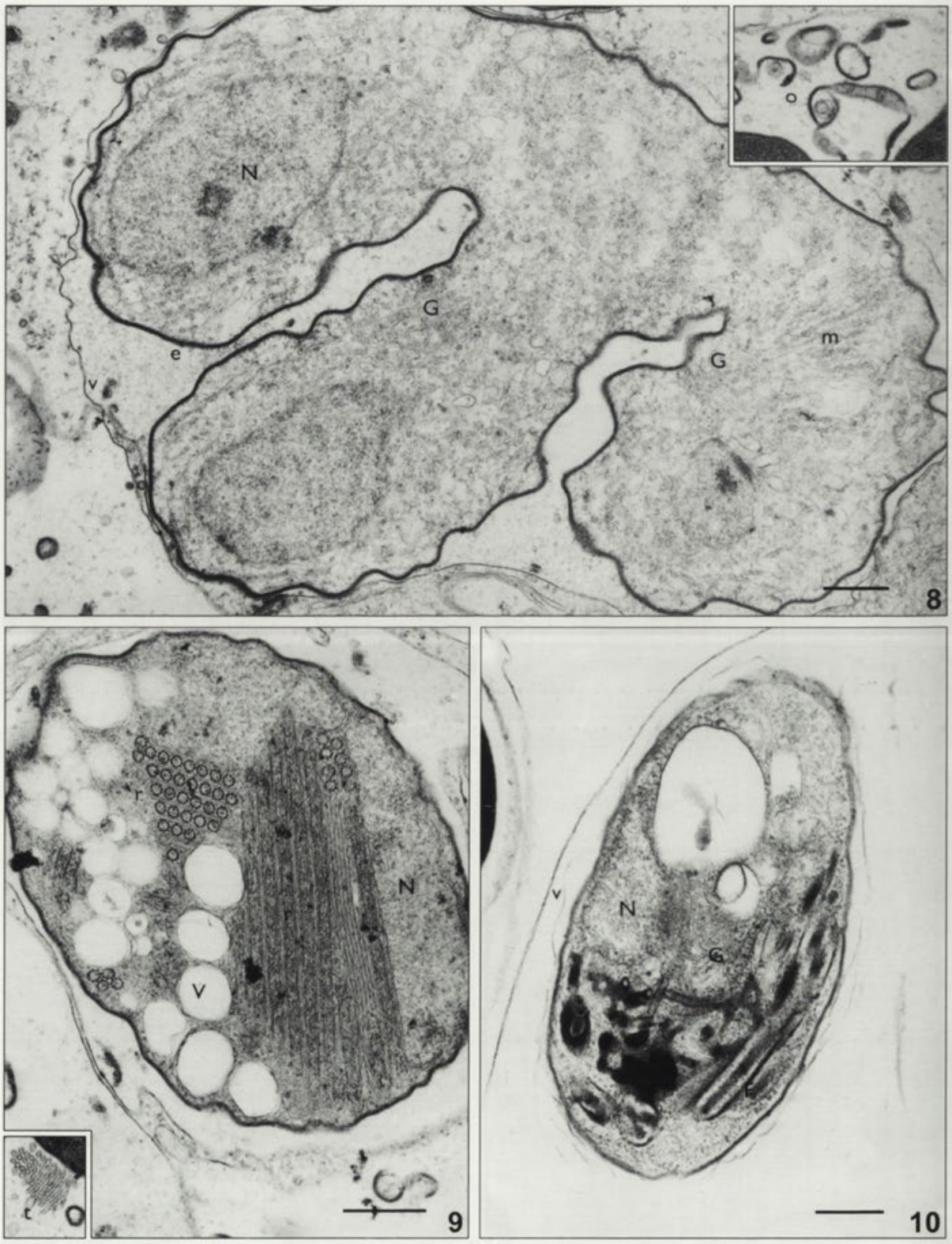

**Figs. 1 a-h**. Light micrographs of developmental stages of *Becnelia sigarae* in merogony, meiogony and sporogony. Symbols used see abbreviations. Scale bar - 5 μm



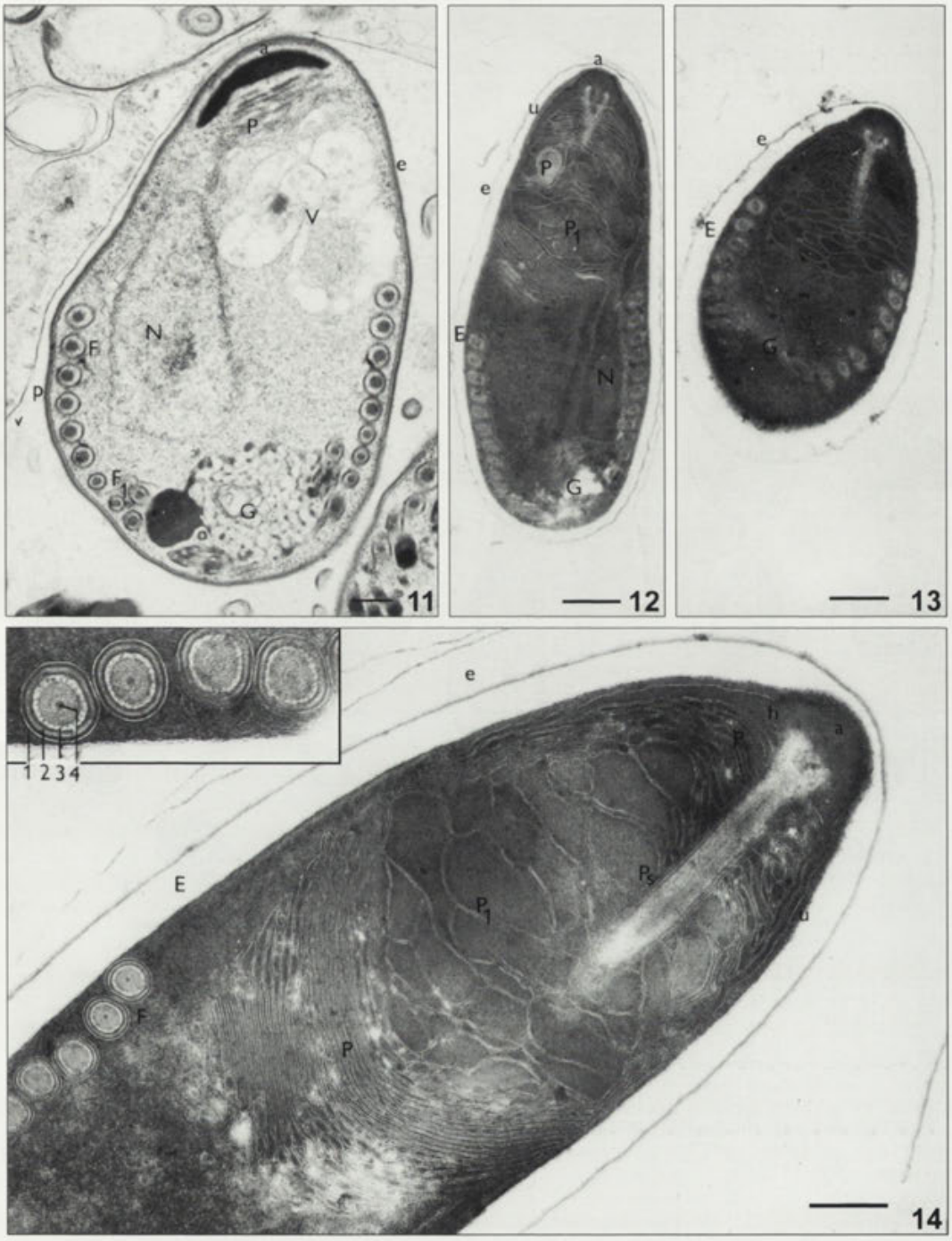

**Figs. 2-4**. Group of developmental stages of *B. sigarae*. **2** - disporoblastic sporophorous vesicle containing 2 spores (D). Close to the sporophorous vesicle (v) there are meronts (M), rosette stage (R), sporonts (Sn) and wrinkled sporoblasts (Sw). **3** - meront of *B. sigarae* with large vacuoles of Golgi (G) close to the nucleus (N) prepared for meiogonial division. **4** - meront with two adhering nuclei (N), exhibited a nucleolus (n). The cytoplasm is enclosed in the thin plasmalemma (p). Scale bars - **2** - 2 μm; **3** - 500 nm; **4** - 1 μm




**Figs. 5-7**. **5** - meronts (M) with thin plasmalemma, early sporont (Sn) without sporophorous vesicle and sporoblasts (Sb) within the sporophorous vesicle (v) with thickened plasmalemma (p) and system of multiple membranes (m). Wrinkled sporoblast with thickened plasma membrane and secretion tubules (Sw). **6** - sporont of the meiotic series. Its nucleus contains multiple synaptonematic complexes (sc). In the cytoplasm, enclosed in a thickened plasmalemma (p), there are several Golgi tubules (G). **7** - sporont with thickened plasmalemma (p), multiple lamellae of the endoplasmic reticulum (er) melting into a system of multiple smooth membranes. Scale bars - **5** - 2; **6** - 1 μm; **7** - 500 nm




**Figs. 8-10**. **8** - part of a sporogonial rosette of *B. sigarae* with nuclei (N) in finger - like buds. Close to the Golgi (G) there are system of multiple membranes (m). Inset: granular secretions (o) appearing in the episporontal space. **9** - sporont with multiple vacuoles (v) and strands of polysomes (r) adhering to the nucleus (N). Inset: tubular secretions (t). **10** - sporophorous vesicle (v) with immature spore. The Golgi (G) is connected with a immature polar filament (F). Scale bars - **8-10** - 500 nm




**Figs. 11-14**. **11** - young spore in the sporophorous vesicle (v) before formation of the endospore. The spore wall is formed from the plasmalemma (p) and an exospore (e) incrusted with electron - dense material. Flat anchoring disc (a) without lateral protrusions. Anterior polaroplast (P) is formed, the central and posterior part not yet constructed in a system of vacuoles (V). Polar filament in 6 broad (F) and 4 narow coils ($F_1$). On one side the Golgi system (G) is connected with the end of the filament, on the other side forming the metachromatic body of the posterosome. **12** - secondary (persistent) mature spore with internal structures. The polar filament (F) is fixed with anchoring disc (a) and prolonged with the umbrella (u) at the apical pole. Golgi system (G) is located near by the nucleus (N). Polaroplast bipartite, with lamellar parts (P) and with central located broad chambers ($P_1$). The spore wall is composed from the exospore (e) and a thick endospore (E). **13** - primary (early) spore with narrow apical end, thick endospore (E), thin exospore (e) and polar filament coiled in 4+3 turns close to the Golgi (G). **14** - anterior end of the secondary (persistent) spore with detailed view of the anchoring disc (a). The umbrella (u) connected with disc in a hinge (h) covers the lamellar polaroplast (P) which encloses the broad chambers of the polaroplast ($P_1$). The spore wall is composed from the outer exospore (e), middle endospore (E) and a inner plasmalemma. The mature polar filament is enclosed in a polar sac (Ps). Inset: cross section of the polar filament with four layers. Scale bars - **11** - 200; **12** - 500; **14** - 200 nm; **13** - 1 μm

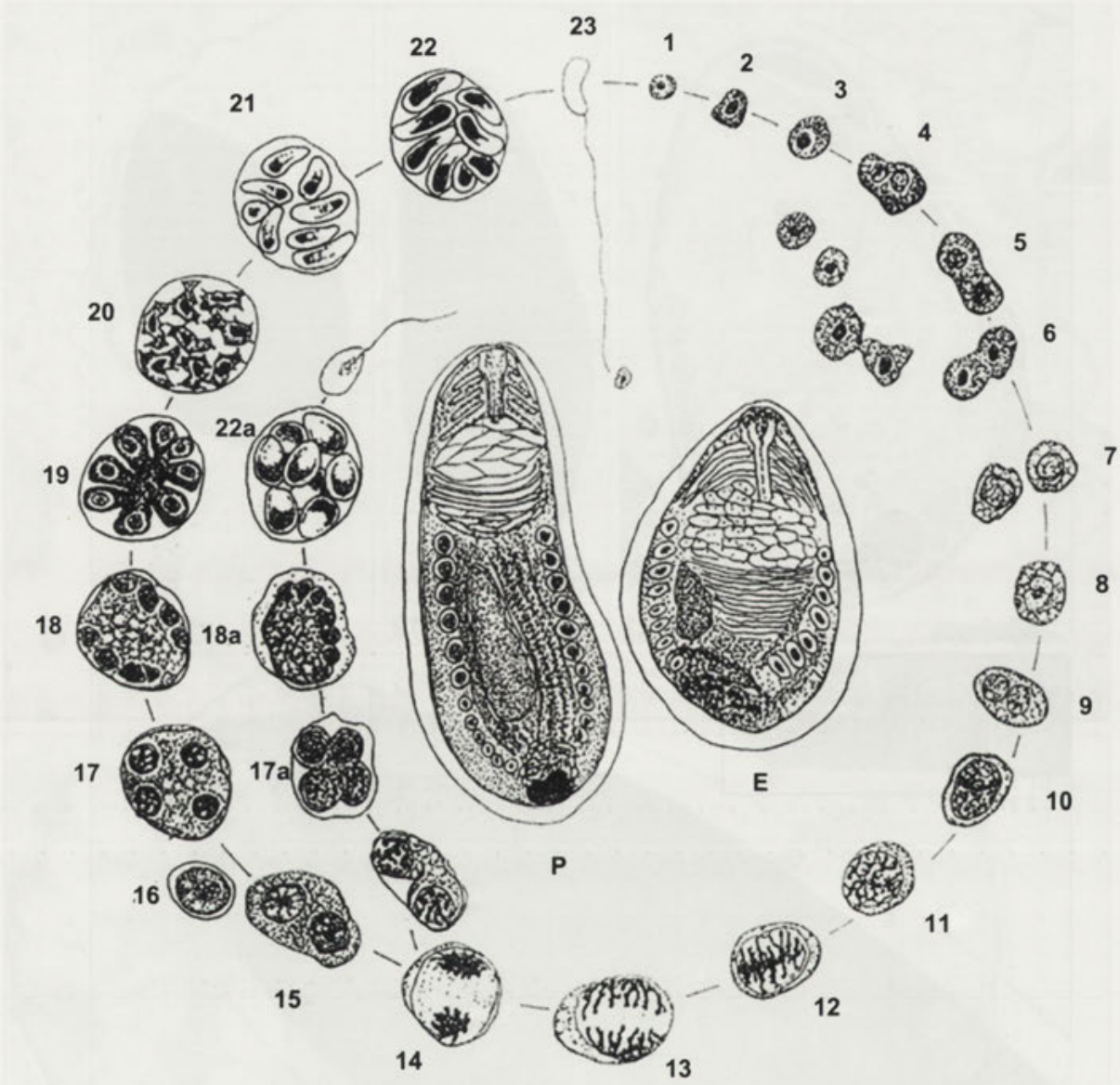



**Fig. 15**. The proposed life cycle of *Becnelia sigarae* (1) Gamet (planont). (2 - 6) Merogony. (7 - 14) Meiogony. (15 - 20) Sporogony, persistent series. (18) Plasmodium. (19) Rosette. (20) Crumpled sporoblasts. (21) Sporoblasts with metachromatic granule. (22) Mature sporophorous vesicle with octospores. (23) Mature spore. (16a - 22a) Sporogony of the early series. P - secondary (persistent) spore, E - primary (early) spore

equally rounded ends, 5 ± 0.5 x 2.5 ± 0.5 µm, was the final product of the sporogony. The spores represented in the evaluated group 71% of all stages. With the same morphology of sporogony we found octosporous pansporoblasts with shorter, broader spores measuring 4 x 2.5 - 3 µm (Fig. 1 - O) which probably represented the „early" spores extruding their filaments in the host and injecting the germs into further tissues to spread the infection in the host. Rare were disporal sporophorous vesicles (Fig. 1 - D), 0.4% of all with spores of irregular size. They eventually represented teratospores varying in shape.

### Electron microscopy

In infected host cells the vegetative stages were common in groups which were formed by dividing merogonial stages. Meronts (Figs. 2-4) were enclosed in a thin plasmalemma (p). In the cytoplasm containing many free ribosomes there were multiple vacuoles and circular lamellae of the rough endoplasmic reticulum. Several groups of vacuoles and tubular formations were part of the Golgi system (Fig. 3). The uninucleate meronts were undergoing a final nuclear division and their nuclei remained sticking together and formed diplokaryons (Fig. 4). These nuclei grow in size, and electron - dense nucleoli (n) appeared to indicate the initial phase of meiotic division. The nuclei of the meiotic series were large and synaptonematic complexes (Fig. 6) signalized the meiotic chromosomes. The plasmalemma of these stages was already thickened but the sporophorous vesicle was not yet formed. At that stage the system of parallel lamellae of the ER disappeared and was transformed in a vacuole with multiple smooth membranes (Fig. 7). With further division of nuclei in the sporonts, the sporophorous vesicle was extended and it enclosed in subsequent divisions a sporogonial plasmodium with two, four and eight nuclei. The individual nuclei were located at the outer wall of the plasmodium and protruded as a finger - like buds forming a rosette (Figs. 2, 8). The buds separated and formed „crumpled" sporoblasts with poor differentiation of the interior where the polar filament and columns of ribosomes were formed. During this stage minute tubules of secretions were formed in the episporontal space between sporoblasts and wall of the sporophorous vesicle. The formation of the polysomes with long spiral coils of ribosomes (9 in each turn) during spore formation was observed (Fig. 9).

With the deposition of the thin electron - lucent endospore the crumpled wall of the sporoblast was smoothened and the internal structures of the spore were formed. In young spores (Fig. 11) the polaroplast was formed from two parts: the anterior lamellar part (P) and the vacuolated central part (V) with broad chambers. The lamellar part enclosed the vacuolated part again at the posterior part of the polaroplast (Fig. 14). One single elongate nucleus was located in the central part. The anisofilar polar filament was apically fixed in a flat anchoring disc (a) and posteriorly coiled in 9 - 10 turns with 5 - 6 turns of larger diameter and 3 - 4 narrower turns. The filament was connected with the tubules of the Golgi system of the posterosome. The large electron - dense granule is the surplus product of the Golgi and can be identified as the red stained metachromatic granule of young spores in Giemsa smears.

In the mature spore (Fig. 12) the anchoring disc ended in a circular hinge, fixed in position by a broad thin umbrella. The polar filament with well differentiated internal structures was fixed in the anchoring disc with an apical calyx and it crossed the polaroplast which filled the first half of the spore. The mature polar filament was coiled in 5 - 6 broader turns (Fig. 12) and 4 - 5 narrower turns. The Golgi complex filled the posterosome, especially its vacuole. The spore wall was composed of a thin electron - dense exospore (Fig. 14) and a rather thick electron - lucent endospore (E) which was attenuated at the apical pole.

Some sporophorous vesicles contained shorter spores measuring 4 x 2.5 - 3 μm. In ultrathin sections they were rare (Fig. 13), more constricted at their apical end. Their endospore was thicker, but indistinct and the polar filament was coiled in 7 - 8 turns. Its anisofilar arrangement was less distinct, the first 4 turns were broader. Some disporous vesicles contained spores of irregular shape, eventually teratospores.

## DISCUSSION

### Infected host

Only one male in the inspected locality was infected with symptoms of reduced transparency of its body and orange colour of the spore masses. The orange colour of an infected organ in rather transparent larvae is known also from infections with *Caudospora* Weiser, 1946 in blackflies where the membranes on the surface of the infected fat body have a brown to red pigmentation (Weiser 1961). This staining is manifested in one and absent in another host and locality. The infection was not in its final phase, there were developmental stages in all inspected parts mixed together with mature spores within the persistent octosporous sporophorous vesicles. The male gonads are the site of infection, microsporidian invaded epithelial cover cells of the testes and some nutritive cells in the germarium and the epididymis, respectively. Other tissues were not found infected.

### General life cycle

The prominent symptom of this infection is a rich representation of vegetative stages, mainly the merogonial sequences. Analogous to merogony we propose the term meiogony for the meiotic sequence and the term meionts

for individual stages involved. It is a series most prominent in *Amblyospora* Hazard and Oldacre, 1975 and *Parathelohania* Codreanu, 1966 and was studied mainly by Debaisieuxi and Gastaldi (1919) and Kudo (1924), recently by Becnel and Andreadis (1999).

The vegetative part of the life cycle in the host was represented in our material by one morphological series with two types of spores. The invasive primary cycle which enables the spread of the microsporidian from its port of entry in the midgut to its tissue of destination is provided by primary (early) spores (Weiser *et al.* 1998, Maddox *et al.* 1999) characterized by a large posterior vacuole when fresh and by differences in ultrastructure of the polar filament. In our material the primary spores are shorter and differ in the number of turns of the polar filament. There is no clear evidence of the anisofilarity except that the four first cross sections are more regular and separate of the rest. The sequence ending with disporous vesicles can be explained as a teratological type of the development. In some cases primary spores may be present as empty spores in the invaded tissue (Weiser *et al.* 1999). In our case an analogous situation is in some sections (Fig. 2) where a sporophorous vesicle with two spores is cut transversally. The spore is too dark to find any details defining it as a primary spore except the constricted apical pole.

**Merogony of the secondary type**

The distribution of germ cells, gamonts, from empty spores may not be very rich in the invasion of the target tissue and such cells analogous to gamets presented in *Edhazardia aedis* (Kudo, 1930), Becnel, Sprague and Fukuda 1989 (Becnel and Andreadis 1999) can be recognized in light microscope with difficulties (cells indicated as g in Fig. 1a). Also the first merogonial series with round stages with dense cytoplasm and minor compact nuclei is not very rich and it is not the series which provides maximum reproduction of the parasite, as it is in *Nosema* Nägeli, 1857 or *Vairimorpha* Pilley, 1976 species. This is well shown in the review of stages of the cycle in the counted 2000 organisms.

The series of meiotic stages (meiogony), typical for *Amblyosporidae* Weiser, 1977 is characterized by the large nuclei and does not participate intensively in the multiplication of the pathogen. But it is a series where karyogamy proceeds rather slowly and therefore it is so common in all smears. During the sporogony 8 spores are produced from each single sporont. The sporophorous vesicle is formed around each sporont as soon as its plasmalemma is thickened. Its origin could not be identified in our material. A step which can be identified in each microsporidian is the stage of the crumpled sporoblast which indicates the short period of first formation of the electron - lucent endospore, when the wall is impermeable for fixation and this causes compression of the content of the sporoblast. The formation of the thickened wall of the sporont begins during the last meiotic changes of the nucleus (Fig. 6). During the crumpled stage there are tubules of electron - dense material released from the forming sporoblast and are resorbed back during the early spore stage.

The reason and fate of the multiple smooth lamellae is not clear. They may eventually be connected with the preparation of the polar sac connected with the active Golgi system and formation of the polar filament. In a rather teratological sporoblast (Fig. 10) it is evident that the parts of the filament are supported by the production of the material in the tubules of the Golgi system. In other section the system of membranes is located close to the nucleus of the bud of the rosette.

The secretions are not very rich in this microsporidian. The spore formation is connected with the formation of masses of ribosomes which are fixed together in columns of polysomes, usually with 9 ribosomes in one circle. The bipartite polaroplast of the mature spore is composed of the anterior lamellar part and a centrally located system of broad chambers. This arrangement does not fit precisely to any type offered in the study of Larsson (1986) but may eventually represent the early step in formation of the helicoidal polaroplast of nosemospores in *Parathelohania* species. The polar filament is anisofilar from its first formation. The electron - dense central chord is in its axis and is fed by material from the Golgi system with evidently connected tubules.

The mature polar filament is organized in 4 principal layers (Vávra 1976, Vávra and Larsson 1999). In the well fixed filament in transversal sections we find four distinct layers and three interspaces. The surface layer 1 is the membrane of the former polar sac, tightly adhering to the filament. The layer 2 is the electron - dense smooth cover which is the transport channel after inversion of the polar tube. In the layer 3 we find the electron - lucent layer, usually amorphous. In well fixed material a longitudinal string of electron - lucent spherical or oval granules forming 12 longitudinal microcylinders was found by Liu and Davies (1973) and 18 subunits were described by Canning and Nicholas (1974). The number

of the longitudinal columns of electron - lucent granules in our microsporidian varied from 22 to 24 and the number of granules is reduced in the narrow turns.

The metachromatic red granule in Giemsa stained smears characterizing the posterosome (Weiser and Žižka 1975) in immature spores is the surplus of product of the Golgi system (the electron - dense mass connected with Golgi tubules).

The microsporidia known from European water Heteroptera do not have any morphologically rich sequence of meiogony. The spores of *Toxoglugea gerridis* and *T. mercieri* have horseshoe -like shape (Poisson 1941, Jírovec 1936) and *Chapmanium nepae* has minute spores 2 - 3 x 1.4 - 1.8 µm in navicular sporophorous vesicles (Hazard and Oldacre 1975). Lipa (1966) mentioned only scarce vegetative stages. *Thelohania veliae* had rather long oval spores (4 x 9 - 11 µm) or broad spores (5.5 - 7 x 7 µm) within octosporous sporophorous vesicles, without distinct merogonial stages (Poisson 1928).

Among microsporidia infecting freshwater insects prominent series of meiotic stages, anisofilar polar filaments and alveolate polaroplasts are characteristics of the members of the family *Amblyosporidae* (Sprague *et al.*,1992). As polymorphic microsporidia they have prominent meiogonial series and anisofilar polar filament in uninucleate thickwalled octospores in larval mosquitoes and the alveolate or helicoidal polaroplast in the thinwalled binucleate spores in adult mosquitoes. Octospores of the microsporidian in *S. lateralis* are thinwalled and uninucleate and the vegetative stages include distinct meiotic series. Therefore we range *B. sigarae* in *Amblyosporidae* but broad chambers we propose new genus *Becnelia* with following characteristics:

***Becnelia* gen. n.**

Microsporidian infecting freshwater arthropods. Schizogony with uninucleate meronts and prominent meiogony with diplokaryotic stages. Sporonts closed in sporophorous vesicles form octosporous plasmodia dividing in a rosette into 8 sporoblasts and spores. Octosporous persistent sporophorous vesicles contain thinwalled elongate or oval uninucleate spores. Polar filament anisofilar. Polaroplast with centrally located broad chambers enclosed in the lamellar parts. Early spores more rounded, with shorter polar filament. Parasites of gonads of aquatic insects.

***Becnelia sigarae* sp. n.**

With characteristics of the genus. End of meiogony and first part of sporogony with thickened plasmatic membrane forming in sporogony a persistent sporophorous vesicle. Minute secretion tubules are resorbed during spore formation. Spores elongate or oval, with rounded ends, slightly curved, 5 ± 0.5 x 2.5 ± 0.5 µm, with anisofilar filament coiled in 9 - 11 turns: 5 - 6 turns thicker, 4 - 5 turns narrower. Early spores 4 x 2.5 - 3 µm, more constricted at the apical pole, filament coiled in 7 - 8 turns.

Site of infection: testes in male.

Host and locality: water boatmen, *Sigara lateralis* Leach, 1817 (Heteroptera: Corixidae), temporary pool near Bavorov, South Bohemia, Czech Republic.

Type material: collection of authors.

Etymology: the generic name is dedicated to J. J. Becnel specialized in research on *Amblyosporidae*.

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Short Communication

# Cytoskeletal Connections between the Nucleus and Cell Cortex in *Amoeba proteus*: A Scanning Electron Microscope Study

**Anna WASIK, Lucyna GRĘBECKA and Andrzej GRĘBECKI**

**Department of Cell Biology, Nencki Institute of Experimental Biology, Warszawa, Poland**

**Summary.** Scanning electron microscopy of *Amoeba proteus* cells, fractured by micromanipulation after fixation and drying, reveals cable-like strands intertwining around the surface of the nucleus. Some strands leave the nucleus, cross the fluid endoplasm and reach the ectoplasmic cortical gel built of F-actin network. These strands contain large F-actin bundles, and are non-permanent structures. They control the nucleus drift forwards with the endoplasmic flow, by anchoring it periodically to the ectoplasmic cylinder.

**Key words:** actin filament bundles, actin filament strands, *Amoeba proteus*, cell nucleus.

## INTRODUCTION

Microfilamentous structures associated with the nuclear envelope have been observed in a variety of cells. It was suggested that they are involved in the changes in shape and size of nuclei, their movements, connections with other organelles and other cytoskeletal structures, and may play a role in the nuclear division. In the metazoan tissue cells filaments were found both inside the nuclei (Nakayasu and Ueda 1985, Milankov and De Boni 1993, Sauman and Berry 1994, Parfenov *et al.* 1995) and around them (Svitkina *et al.* 1984, Henderson and Locke 1992, Clubb and Locke 1996).

Address for correspondence: Anna Wasik, Nencki Institute of Experimental Biology, Polish Academy of Sciences, Department of Cell Biology, ul. Pasteura 3, 02-093 Warszawa, Poland; Fax: (4822) 822 53 42; E-mail: annaw@nencki.gov.pl

In *Amoeba proteus* the fibrillar material associated with interphasal and mitotic nuclei was identified, in the intact cells and in the isolated nuclei, by staining with FITC-conjugated phalloidin and antibodies against actin and myosin (Pomorski and Grębecka 1993, 1995; Grębecka *et al.* 1999; Pomorski *et al.* in press). Recently, a transmission electron-microscopy study (Grębecka *et al.* 1999) has shown that filamentous actin is present in these cells on the cytoplasmic side of the nuclear envelope as well as in the peripheral karyoplasm.

While the association of nuclear envelope of *A. proteus* with F-actin filaments seems to be well documented, there is only one report (Pomorski and Grębecka 1995) postulating the existence of transient physical links between the perinuclear actin shell and the cytoplasmic F-actin cytoskeleton located in the ectoplasmic cortical gel of amoebae. On the contrary, their endoplasm is generally assumed to be a fluid sol containing only G-actin. In the present study

we try to demonstrate, by scanning electron microscopy (SEM) of fractured amoebae, that the endoplasmic sol may be intersected by organized strands linking the nucleus with the cortical cytoskeleton and/or with other cytoplasmic organelles.

## MATERIALS AND METHODS

The cultures of *Amoeba proteus* strain C were grown in Pringsheim medium at room temperature and fed twice a week on *Tetrahymena pyriformis*. The living cells were observed in a Biolar differential interference microscope (PZO, Warszawa). For fixation the cell samples were pipetted into 3.5% paraformaldehyde in phosphate buffered Pringsheim medium. Some of them were stained with 1% FITC-conjugated phalloidin (Sigma, St. Louis) and examined in a confocal laser scanning microscope (Molecular Dynamics CLSM Phoibos 1000, based on Nikon Optiphot microscope). The others were processed for SEM observations: dehydrated through a graded series of ethanol and acetone, then dried by the $CO_2$ critical point method. These cells were fractured by micromanipulation and then coated with carbon and gold. Morphological and ultrastructural details were examined in a JEOL 1200 EX transmission electron microscope equipped with ASID 19 scanning attachment operating at 40 KV.

## RESULTS AND DISCUSSION

Christiani *et al.* (1986) using the transmission electron microscopy and replica techniques precisely examined the extracted cytoskeleton of normal, locomoting *Amoeba proteus*. They revealed that it is composed of at least 4 types of filaments; the cell structures were correctly preserved, which indicated that drastic premortal alterations of the cytoskeleton were avoided. However, these authors were merely concerned about the locomotor cortical cytoskeleton.

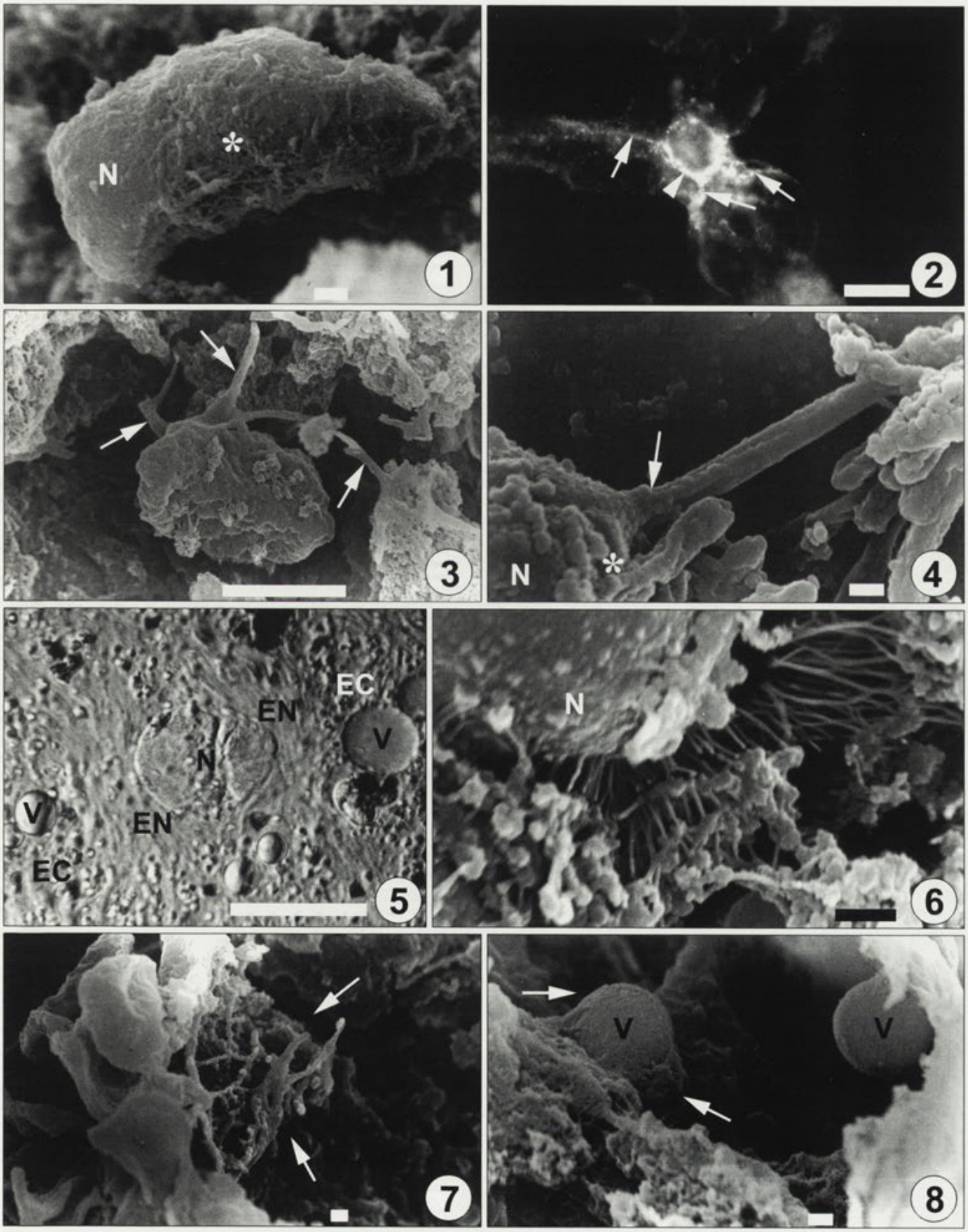
Our main purpose was to detect the cytoskeletal connections between the nucleus and other structures in a moving amoeba. The cells fixed, fractured and examined in SEM demonstrated networks of strands twisting closely around the outer nuclear surface (Fig. 1). We suppose that they are fibrillar structures because, as it was previously immunochemically shown (Grębecka *et al.* 1999), actin, spectrin and myosin are connected with the nuclear envelope of *Amoeba proteus*. The filamentous state of the perinuclear actin has also been earlier demonstrated by staining nuclei with FITC-conjugated phalloidin (Pomorski and Grębecka 1993, 1995; Grębecka *et al.* 1999; Pomorski *et al.* in press). Very bright fluorescence of phalloidin on the nuclear surface is seen as well in Fig. 2, in the present paper. It allows us to postulate that the strands shown by SEM on the nuclear envelope contain F-actin filaments, probably connected with the nuclear surface by spectrin (Choi and Jeon 1989). Similar results were obtained in fibroblaststs by Svitkina *et al.* (1984).

The SEM images of whole fractured cells of amoebae allow us to localise easily the nucleus among different cytoplasmic structures (Fig. 3). The same Figure demonstrates as well that some cable-like strands leave the nuclear surface (arrows) and run toward various sites in the cytoplasm (arrowheads); they may reach the cell periphery or different organelles. Fig. 4 shows in a higher magnification the base of another strand emerging from the nuclear surface, settled among other strands that form the perinuclear network. The F-actin staining with phalloidin (Fig. 2) reveals similar linearly arranged F-actin bundles running from the nucleus to the peripheral cytoplasm. This allows us to suggest that the organized strands crossing the fluid endoplasmic sol, which are detected by SEM, are actually gel cables formed by parallel alignment of F-actin filaments.

The nucleus of a locomoting amoeba is carried forwards by the endoplasmic flow faster than the cell moves, but periodically it stops for a time needed to keep it at a nearly central position in the cell (Fig. 5). It seems, therefore, that the actin-containing strands only transiently anchor the nucleus to the almost stationary ectoplasmic cylinder of a moving amoeba. Consequently, they may not be the permanent cell structures. As a matter of fact, they were revealed by SEM in about a half of examined cells. Their presence or absence may probably depend, respectively, on the stationary condition or drifting of each nucleus at the moment of fixation.

In a few cases the threads emerging from the nuclear surface had the form of a brush of thinner short bundles (Fig. 6). We also saw such brush-like structures building

**Fig. 1.** Fibrillar network (*) on the external surface of nucleus (N); SEM. **Fig. 2.** F-actin associated with the nuclear envelope (arrowhead) and in the cable-like strands (arrows); FITC-conjugated phalloidin. **Fig. 3.** Cable-like strands (arrows) running from the nucleus toward the neighbouring cytoplasmic structures; SEM. **Fig. 4.** High magnification of the base of a strand (arrow) emerging from the nucleus (N) surrounded by perinuclear cytoskeleton (*); SEM. **Fig. 5.** Immobile nucleus (N) in the flowing endoplasm (EN) and vacuoles (V) in the stationary ectoplasm (EC); differential interference contrast microscopy. **Fig. 6.** Brush of short bundles running from the nucleus (N); SEM. **Fig. 7.** Fragment of fibrillar network (arrows) located beneath the plasma membrane; SEM. **Fig. 8.** Vacuoles (V), left one fastened to the fibrillar network (arrows); SEM. Scale bars: **1, 4, 6-8** - 1 μm; **3** - 10 μm; **2, 5** - 25 μm



bridges between different, membrane-surrounded, cytoplasmic components (not shown). A fragment of the three-dimensional F-actin network associated with the plasma membrane at the cell periphery is seen in (Fig. 7). Fig. 8 shows two vacuoles: one of them (probably the contractile vacuole) is embraced by the adjacent cortical F-actin network, whereas another one (probably a food vacuole) is naked. Actin containing fibrillar structures of comparable dimensions were found in a variety of metazoan cells (Svitkina *et al.* 1984, Bershadsky and Vasiliev 1988, Selchow and Winklbauer 1997, Welnhofer *et al.* 1997, Wulfkuhle *et al.* 1998).

It might be postulated that the huge strands emerging from the nuclear surface and the more subtle fibrillar bundles and networks, seen by us in SEM, have the same actin nature and common origin. Their conformation may be imposed by the rheological conditions. In a migrating amoeba the cytoskeletal bridges are exposed and resist, when anchored, to the shearing force of the endoplasmic streaming. Consequently, the cytoskeletal connections of the nucleus (or of some other floating elements) with the F-actin network in the walls of the ectoplasmic cylinder are periodically extended, internally reorganized by strain, broken and reconstructed. So, actually they can change shape and structure, oscillating between three-dimensional networks, linear filament bundles and massive strands. Probably, both fibrillar forms of the cytoskeleton in *Amoeba proteus* are involved in the autonomous nuclear movements (Pomorski and Grębecka 1995) and, moreover, it should not be excluded that they may control the behaviour of some other membrane-surrounded organelles in the endoplasmic stream.

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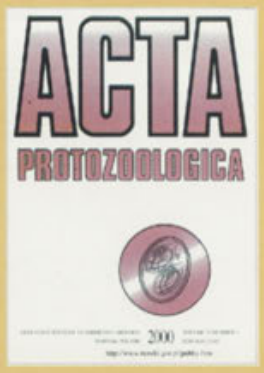


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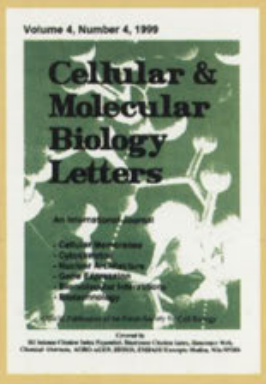


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