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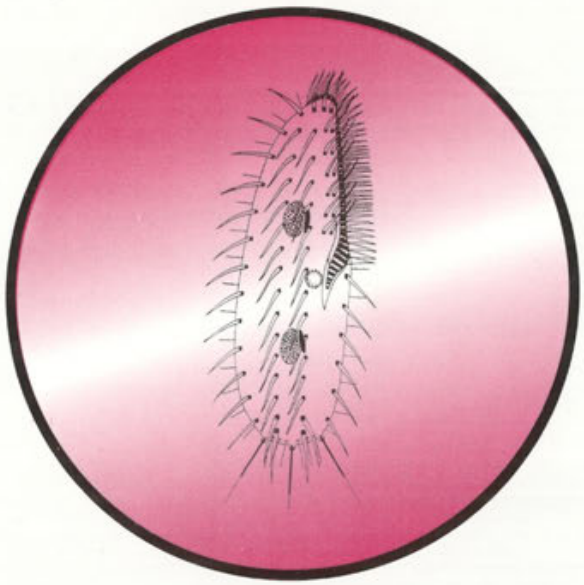

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Reviev article

The Dinoflagellate Nucleus and Chromosomes: Mesokaryote Concept Reconsidered

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Summary. This review considers the literature data which appeared mainly during the latest 12 years. Chromosome structure in dinoflagellates and new models of chromosome organization are reviewed. The chromosomes are no longer considered to be polytene. The existence of eukaryotic-type cell cycle in dinoflagellates contradicts the mesokaryote concept. Mitosis in dinoflagellates (class Dinoflagellatea) is usually extranuclear closed pleuromitosis with the spindle microtubules hidden inside channels which pierce the dividing nucleus, but it can be also closed intranuclear (in *Oxyrrhis*, belonging to class Protalveolatea). Sequencing of both large and small subunit ribonuclear RNA (or the corresponding genes coding for them) has demonstrated late emergence of dinoflagellates among eukaryotes and their clustering with ciliates and sporozoans; the dinoflagellates thus cannot any more be considered mesokaryotes, meant as a sister group to all eukaryotes. The dinoflagellates frequently harbour eukaryotic photosynthetic symbionts which show various degrees of integration into the dinoflagellate cell.

Key words. Dinoflagellates, nucleus, dinokaryon, chromosomes, mesokaryotes, rDNA sequencing.

INTRODUCTION

This review aims to update and supplement Chapter 4 of the author's book (Raikov 1982) on protozoan nuclei. Much new information has been published since the early eighties which brought into question the mesokaryote nature of the dinoflagellates, i.e. that they are in some way intermediate between prokaryotes and eukaryotes (Dodge 1965) or represent a sister group to all extant eukaryotes (Herzog et al. 1984) conserving a number of prokaryotic features. The new evidence accumulated shows rather that the dinoflagellates are eukaryotes which secondarily lost typical histones and thus acquired a resemblance to prokaryotes. This evidence comes mainly from two directions: first, the demonstration of eukaryotic cell cycles in dinoflagellates, and second, the sequencing of ribosomal RNAs or ribosomal genes which proved to be eukaryotic. These aspects will be considered in the present review. Besides this, new data accumulated on chromosome structure in dinoflagellates which refuted the polytenic model of the dinoflagellate chromosome (Haapala and Soyer 1973) which prevailed in the seventies.

Little change occurred in the notion about dinoflagellate mitosis. Dinomitosis can still be considered a variant of closed extranuclear pleuromitosis. However, closed

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intranuclear mitosis has been described in a somewhat aberrant dinoflagellate (*Oxyrrhis*).

Finally, symbiosis of dinoflagellates with various algae usually conserving their proper (typically eukaryotic) nucleus has been further investigated. This type of symbiosis proved to be common among dinoflagellates.

NUCLEUS AND CHROMOSOMES

It is well known that the nucleus of most dinoflagellates, the so-called dinokaryon, displays in the interphase fully condensed chromosomes of highly ordered structure. These consist of many elementary fibrils and show transverse bands where the fibrils proceed across the chromosome, and interbands where the same fibrils form arcs while going from one band into the next (review: Raikov 1982).

A number of recent reviews is available considering the structure of dinoflagellate chromosomes and the impact of their organization on the ideas about phylogeny of these protists: Dodge (1983a, 1985), Spector (1984), Sigee (1986), Desnitzky (1990), Rizzo (1991).

Number of chromosomes

It has already been known in the early eighties (Raikov 1982) that free-living dinoflagellates usually have high chromosome numbers (20-270) while most parasitic ones (*Syndinium, Merodinium, Amoebophrya,* etc.) have few chromosomes (4-8). Such data can also be found in later reviews (Dodge 1985, Sigee 1986, Desnitzky 1990). The number of chromosomes can strongly vary within a single genus, e.g., *Peridinium* (Holt and Pfiester 1982). This indirectly indicates polyploidy of some species with many chromosomes. In the symbiotic dinoflagellate *Symbiodinium,* 26 chromosomes occur (Blank 1987), in two species of *Prorocentrum,* 32 and 132 (Costas and Goyanes 1987).

The nuclear DNA content of dinokarya is not clearly correlated with the number of chromosomes (Desnitzky 1990). It can vary between about 3 pg (in *Amphidinium carterae*) and 200 pg (in species of *Gonyaulax*), i.e. is often unusually high (reviews: Raikov 1989, Desnitzky 1990).

Properties of chromosomes

The dinoflagellate chromosomes have already been known to consist virtually of pure DNA, with no typical histones and very little basic protein of specific composition (reviews: Raikov 1982, 1989). Up to 60% of their DNA is represented by repetitive sequences in *Crypthecodinium* (Allen et al. 1975; review: Dodge 1985), and about 45%, in *Woloszynskia* (Davies et al. 1988). The DNA of the dinoflagellates proved to bind much divalent metal cations, especially Ni, Fe, Cu, Zn (Sigee 1982, 1983; Sigee and Kearns 1982; review: Dodge 1985) as well as Ca and Mg which seem to stabilize the chromosome structure (Herzog and Soyer 1983). Instead of the histones, the chromosome compactness is stabilized by some special kind of RNA which seems to play a structural role (Soyer-Gobillard and Herzog 1985).

The DNA of the dinoflagellates is peculiar in that a large proportion of its thymine (5-methyluracil) is replaced by its analog, 5-hydroxymethyluracil (Galleron 1984, Davies et al. 1988). In *Prorocentrum micans*, this base replaces 63% of thymine (Herzog et al. 1982). This feature is not typical of either prokaryotes or eukaryotes.

The main part of the DNA contained in condensed chromosomes of *Prorocentrum micans* proved to be usual right-handed double helical DNA (B-DNA). However, left-handed or Z-DNA has been found by immunocytochemical methods near the segregation fork of dividing chromosomes and in the NOR area of nucleoli, i.e. near the points where the chromosome is decondensed; Z-DNA possibly facilitates unwinding and replication or transcription (Soyer-Gobillard et al. 1990).

The basic protein(s) of dinokarya constitute only 10-13% of the quantity typical of eukaryotes (Rizzo et al. 1982, Vernet et al. 1990). They are not true histones either, although they include, in *Crypthecodinium,* up to six different fractions with molecular masses comprised between 14 and 17 kDa (Vernet et al. 1990). Little basic protein occurs also in species of *Gymnodinium* (Rizzo et al. 1982, Rizzo and Burghardt 1982). The basic protein of dinokarya also differs from true histones in that it contains cysteine and aromatic amino acids (reviews: Dodge 1985, Desnitzky 1990). However, there is one exception among the dinoflagellates: in the somewhat aberrant *Oxyrrhis marina,* which also shows atypical mitosis (see below), several fractions of basic proteins which correspond to typical histones were found, the proportion of basic protein being higher and reaching half the usual eukaryotic level (Farmer et al. 1983; Li 1983, 1984). In some recent classifications, *Oxyrrhis* is separated from true dinoflagellates into another class or even subphylum, Protalveolat(e)a (Cavalier-Smith 1993, Corliss 1994).

This absence of histones in dinoflagellate chromosomes is known to be correlated with the absence of nucleosome subunits of chromatin (earlier literature re-

viewed by Raikov 1982). The component fibrils of these chromosomes are smooth and have a diameter of 4-6 nm (Rizzo et al. 1982, Rizzo and Burghardt 1982, Costas and Goyanes 1987), i.e. they are about two times thinner than typical nucleosome-studded fibrils of eukaryotes. The occurrence of nucleosomes in the histone-containing *Oxyrrhis marina* is not yet clear: according to Farmer et al. (1983), *Oxyrrhis* has no nucleosomes, but according to Fan et al. (1983) and Li (1983, 1984), "nucleosome-like particles" are present in its chromatin. Positive birefringence is correlated with histone-less organization and absence of nucleosomes in typical dinoflagellate chromosomes (Cachon et al. 1989).

Models of chromosome organization

During the seventies, models postulating polyteny of dinoflagellate chromosomes prevailed, according to which the chromosome would consist of many (several hundred) "genofibrils" (Grassé et al. 1965) or circular chromatids (Haapala and Soyer 1973). However, genetic data and data on the rate of DNA renaturation have demonstrated that at least *Crypthecodinium cohnii* is haploid (Roberts et al. 1974, Allen et al. 1975, Tuttle and Loeblich 1977). 55-60% of the DNA of this species is repetitive and the rest is unique or represented by only a few copies, while the polytenic model predicts that all DNA sequences would be repeated hundreds of times (reviews: Raikov 1982, 1989). All this favours haploidy and uninemic models of chromosome organization in dinoflagellates, although species other than *C. cohnii* may well turn out to be polyploid.

It has previously been supposed that dinoflagellate chromosomes permanently remain condensed both throughout the interphase and during mitosis (see Raikov 1982). Only artificial unwinding was supposed to occur, e.g. during spreading of the chromosomes. Spreading usually leads to transformation of the chromosome into a series of figures of eight formed by two mutually intersecting and oppositely directed bundles of many unit fibrils. At the ends of the stretched chromosome, the bundles are continuous forming a loop, so in fact there is only one circular (toroidal) fibrillar bundle twisted upon itself (Haapala and Soyer 1973, Oakley and Dodge 1979). However, it has been shown that at least in *Peridinium cinctum, Crypthecodinium cohnii* and *Prorocentrum* there exists a phase of chromosome uncoiling during interphase, which corresponds to the S period of the cell cycle (Spector et al. 1981, Spector and Triemer 1981). The chromosome uncoils into a "chromonema" which consists of several parallel core fibrils 9 nm thick and two helical arrays of fibrils 2.5 nm thick winding around the core fibrils. The outer helix additionally bears granules 9 nm in size and is connected with the inner helix by 2.5 nm connecting fibrils. A phase of natural unwinding of the chromosomes was also observed in *Gonyaulax* by Donner and Rensing (1984) and *Symbiodinium* by Blank (1986). It is however uncertain why this structure (two types of fibrils, etc.) was never seen in spread chromosomes and how the "chromonema" is packed to form the condensed chromosome.

Another uninemic model of chromosome structure in dinoflagellates was put forward by Oakley and Dodge (1979). Using chromosome spreading in *Amphidinium,* they obtained the usual series of eights indicating that a toroidal bundle of many unit fibrils is twisted upon itself. But, unlike polytenic models, they suppose that the toroidal bundle consists of only one DNA molecule (unit fibril) looped around many times, rather than of many circular DNA molecules.

A third model of chromosome organization considers the dinoflagellate chromosome as a liquid crystal of cholesteric type formed by deproteinized DNA. The same type of liquid crystal can be obtained in vitro from concentrated solutions of DNA (Livolant and Bouligand 1978, Livolant 1984). The twisted toroidal bundle seen in spreads is considered an artefact (Livolant and Bouligand 1980). The native dinoflagellate chromosome is considered as a stack of flat liquid crystals in which the only DNA filament is packed in zigzag manner and also connects one disk with the next one where the direction of the zigzag is altered by a constant angle (Bouligand et al. 1968, Livolant and Bouligand 1978, Livolant 1984). Mathematical analysis shows that this model correctly predicts the observed pattern of arrangement of the DNA filaments in sections through the chromosome. This has been confirmed also in cryosections (without chemical fixation) of *Prorocentrum* chromosomes, which procedure is likely to preserve the native configuration of the DNA fibrils (Gautier et al. 1986).

For the time being, it is difficult to decide which model of dinoflagellate chromosome architecture is correct. Only polytenic models are clearly out of date, whereas uninemic models are favoured.

Nucleoli

The nucleoli are known to be formed in dinoflagellates on locally unwound chromosomes, so that the DNA fibrils spread out inside the nucleolus and constitute the nucleolar organizing region or NOR (review: Raikov 1982). In *Prorocentrum micans,* the NORs occur on

several chromosomes but only one common nucleolus is formed (Gavrila and Mihaescu 1986, Soyer-Gobillard and Géraud 1992). In situ hybridization with rRNA confirmed in the same species that rRNA coding sequences of DNA are localized inside the nucleolus, i.e. on the decondensed portions of the respective chromosomes (Géraud et al. 1991).

The nuclear envelope

Little new became known about the structure of the nuclear periphery in dinoflagellates. It has previously been known that the dinoflagellate nucleus is usually limited by a conventional two-membraned and pore-bearing envelope; however, in *Noctiluca* the nuclear envelope evaginated in form of numerous pore-bearing vesicles, which greatly increased the nuclear surface (reviewed by Raikov 1982).

The colonial (four-celled) dinoflagellate *Polykrikos* shows a strongly developed nuclear lamina inside the envelope which also presents evaginations in form of ampullae carrying numerous pore complexes (Bradbury et al. 1983). As to the portions of the nuclear envelope which are not evaginated, they seem to lack pores, as in *Noctiluca.*

CELL CYCLES

The problem of cell cycles in dinoflagellates has been recently reviewed by Desnitzky (1988). The dinoflagellates were previously thought to lack subdivision of the interphase into discrete periods (presynthetic or G1, synthetic or S, and postsynthetic or G2). The DNA synthesis was thought to proceed in their nuclei continuously, not only during interphase but also during mitosis; at most, discrete DNA synthesis in them would be induced by photoperiodism (review: Raikov 1982). This would then constitute a prokaryotic character of the dinoflagellates.

Most recent studies demonstrate however that the cell cycle of dinoflagellates is typically eukaryotic: it includes discrete G1, S, G2 and M (mitosis) periods. This was described as early as in 1970 in *Peridinium trochoideum* and *Gonyaulax tamarensis* (Polikarpov and Tokareva 1970). In *Amphidinium,* Galleron and Durrand (1979), using labelled thymidine, found an eukaryotic cell cycle with discrete periods S, G2 and M. In *Peridinium cinctum,* a distinct S-phase has been observed using labelled thymidine incorporation; this corresponded to chromosome unwinding, as described above (Spector et al. 1981). Measuring DNA in mass cultures of six marine dinoflagellates, Karentz (1983) reported in all of them the existence of a discrete S phase.

In *Prorocentrum,* there is a long (about 120 h) G1 period, followed by a less than 4 hours' S period, and a G2+M phase of about 8 h (Bhaud and Soyer-Gobillard 1986). According to observations of temporary unwinding of the chromosomes, which probably corresponds to S, eukaryotic cell cycle also occurs in *Symbiodinium* (Blank 1986).

A more complex life cycle occurs in *Crypthecodinium.* It includes two consecutive cell cycles, one with motile G1 cells which then encyst, and the other entirely in cysts; the two cycles are different as to the length of the component periods (Bhaud et al. 1991, 1994). The first cycle has a long G1 period (about 6 h), an S period of about 1.5 h, and G2+M of about 1 h 20 min; the second cycle has a short G1 period and G2+M of about 2 h (Bhaud et al. 1991). A more refined study (Bhaud et al. 1994) has shown that the first cycle comprises a distinct, however short G2 period (0.5 h) and permitted to give the detailed timing of the second cycle (G1, 0.5 h; S, 1.5 h; G2, 2 h; M, 2 h). Four motile cells are released from the reproduction cyst; in some cases, there are three rather than two divisions in the cyst, giving rise to eight cells (Bhaud et al. 1991).

MITOSIS

The mitosis has already been well known in both free-living and parasitic dinoflagellates by the early eighties (for review see Raikov 1982). It is a variant of closed extranuclear paramitosis characterized by an entirely cytoplasmic spindle (see also Raikov 1994). In free-living forms, the spindle is subdivided into several or many bundles which are enclosed in cytoplasmic tunnels piercing the nucleus. There are both non-kinetochore and kinetochore microtubules inside the tunnels. The kinetochores of the chromosomes are built into the nuclear envelope lining the tunnels and a single microtubule attaches to each daughter kinetochore from inside of the tunnel. In most parasitic forms (*Syndinium* and alike), there is only one tunnel through the nucleus, and centrioles are found at the poles of the spindle (review: Raikov 1982).

In the last years, most studies confirmed these observations. Spector and Triemer (1981) observed cytoplasmic tunnels, kinetochores and their division in two before separation of daughter chromosomes in *Peridinium*

and *Polykrikos*. Fritz and Triemer (1983) found essentially the same in *Prorocentrum minimum*, where formation of tunnels occurs before the appearance of microtubules in them and consequently the microtubules do not depress the nuclear envelope as was previously supposed.

Barlow and Triemer (1988) found that only two tunnels are formed through the dividing nucleus of *Amphidinium carterae*. They have demonstrated by immunofluorescence of tubulins that all microtubules are enclosed into tunnels and also shown electron microscopically that the central spindle in reality consists of two interdigitating half-spindles; they also found a kind of spindle pole bodies at the poles of the mitotic figure to which the microtubule bundles converge. According to their data, chromosome separation occurs in dinoflagellates solely due to the elongation of the central spindle (in result of mutual sliding of the half-spindles), the length of kinetochore microtubules remaining constant.

This rather uniform picture is somewhat disturbed by the finding that in the aberrant dinoflagellate *Oxyrrhis marina*, which also possesses histones (see above), the mitosis follows quite another pattern. Mitosis is here closed intranuclear and proceeds inside an intact nuclear envelope, although the central spindle is subdivided into several bundles of microtubules, each with its own polar plaques built into the nuclear envelope (Triemer 1982). Perhaps *Oxyrrhis* is not a dinoflagellate at all or should be singled out at a high level within the dinoflagellates (Triemer 1982). In fact, recent classifications separate *Oxyrrhis*, together with *Colponema*, into another subphylum, Protalveolata (Cavalier-Smith 1993), or class, Protalveolatea (Corliss 1994), the "true" dinoflagellates constituting, respectively, subphylum or class Dinoflagellat(e)a. As in true dinoflagellates, the chromosomes separate in *Oxyrrhis* due to mutual sliding of non-kinetochore microtubules rather than due to shortening of kinetochore microtubules. The nucleus of *Oxyrrhis* presents deep invaginations of the nuclear surface, but these have nothing to do with mitosis or with formation of cytoplasmic tunnels through the nucleus, as was previously supposed (Cachon et al. 1979). The observations about the occurrence of an intranuclear spindle subdivided in many subspindles have been confirmed in *Oxyrrhis marina* by Gao and Li (1986). They also observed that the numerous subspindles terminate against polar plaques at the level of the nuclear envelope.

SEQUENCING OF RIBOSOMAL RNAs

Since the early eighties, an entirely new method of independent assessment of the phylogenetic relations of various protists has been applied to the dinoflagellates: sequencing of rRNAs or of the respective rRNA genes.

One of the first studied was the relatively small 5 S rRNA. Its partial sequencing was first carried out by Hinnebusch et al. (1981) who found that this RNA was more eukaryotic than prokaryotic (it lacked certain highly conserved prokaryotic residues) and that dinoflagellates (*Crypthecodinium*) emerged rather late in evolution, well after the emergence of fungi. Kumazaki et al. (1983) found on the basis of 5 S rRNA sequences that *Crypthecodinium* clusters with the ciliates and emerges rather late, well after the animal-plant separation and even later than the emergence of euglenids and kinetoplastids. Krishnan et al. (1990), using a new algorithm for 5 S rRNA sequence comparison, also demonstrated that the dinoflagellates are related with the ciliates.

Less clear results were obtained with 5.8 S rRNA but the very existence of this RNA indicates the eukaryotic nature of the dinoflagellates (prokaryotes lack this RNA); here also, *Crythecodinium* emerges later than fungi (Hinnebusch et al. 1981). This incertitude induced Maroteaux et al. (1985), who sequenced the 5.8 S rRNA gene of *Prorocentrum*, to think that dinoflagellates diverged early in eukaryote evolution and could represent a sister group to all extant eukaryotes, an idea proposed on the basis of nuclear phenotypic characters by Herzog et al. (1984).

More informative proved to be the small-subunit rRNA of dinoflagellates (usually about 17 S). Herzog and Maroteaux (1986) cloned and sequenced the respective gene in *Prorocentrum*. The tentative secondary structure of the 17 S rRNA was mostly eukaryotic, but the primary structure was still consistent with the idea that dinoflagellates were a sister group to all other eukaryotes; in fact, *Prorocentrum* diverged almost in the same time as *Didymium* and earlier than yeasts and higher plants. No comparison with ciliates was done in this work.

Schlegel (1991) extensively reviewed the phylogenetic relations between various protists on the basis of existant 17 S rRNA sequences. According to all methods of comparison of the nucleotide sequences, the dinoflagellates (*Prorocentrum* and *Crypthecodinium*) emerge rather late in evolution and form a clear cluster with ciliates (Ciliophora) and sporozoans (Apicomplexa). This is more consistent with the view that dinoflagellates

are eukaryotes which secondarily acquired some prokaryote-like characters, such as absence of histones and nucleosomes.

Cavalier-Smith (1993) carried out an extensive comparison of the published and newly obtained sequences of the small-subunit rRNA in 150 eukaryotes. Data on five dinoflagellates were included: *Prorocentrum micans, Crypthecodinium cohnii, Alexandrium (Gonyaulax) tamarense, Amphidinium belauense,* and *Symbiodinium* sp. They all tightly clustered together, indicating their monophyly, and also clustered with Ciliophora and Apicomplexa (coccidians, haemosporidians and piroplasmids), the whole branch emerging rather late in evolution. This relationship between dinoflagellates, ciliates, and apicomplexans is not surprising, since they share many phenotypic characters as well and can be considered a monophyletic group, the Alveolata (Cavalier-Smith 1993, Corliss 1994).

The clustering of ciliates with dinoflagellates and apicomplexans on the basis of small-subunit rRNA sequence has also been observed by Hammerschmidt et al. (in press). Sequences of four dinoflagellates, four apicomplexans, and many ciliates were used in this work primarily centred on the ciliates.

Finally, dinoflagellate large-subunit rRNA (23-26 S) has also been used for phylogenetic purposes. Partial sequencing of the highly conserved 5'-end of this RNA has shown a high homology of the nucleotide sequences between dinoflagellates (*Prorocentrum*) and ciliates, which form a definite cluster emerging rather late in eukaryote evolution (Baroin et al. 1988, Qu et al. 1988). Complete sequencing of the large-subunit rRNA of *Prorocentrum* has confirmed high homology between it and that of the ciliates; it also showed similarities with the large rRNA of yeasts (Lenaers et al. 1989).

Additional evidence about molecular similarity of the dinoflagellates with eukaryotes may be obtained from studies of the small nuclear RNAs. Their presence is an eukaryotic feature of the dinoflagellates. The U1-U6 snRNAs proved to be homologous to those of the rat (Reddy et al. 1983). Among them, the U5 small nuclear RNA has been sequenced and proved to be more similar to U5 of higher eukaryotes (rat, chick, man) than to that of lower eukaryotes (Liu et al. 1984).

EUKARYOTIC SYMBIONTS

It has been already known in the early eighties (review: Raikov 1982, p. 265), that dinoflagellates may have intracellular eukaryotic symbionts conserving their own nuclei. The story began with description of "binucleate" dinoflagellates, possessing an eukaryotic-type nucleus besides of an usual dinokaryon. This was at first suspected to be a case of true nuclear dualism, but it rapidly became known that not only the eukaryotic nucleus had symbiotic origin, but that some cytoplasmic organelles of the symbiont, such as plastids and mitochondria, were also retained. Histones and nucleosomes were found only in the symbiont nucleus (Rizzo and Cox 1977, Rizzo and Burghardt 1980, Rizzo 1982, Shupe and Rizzo 1983, and others).

More recently, Dodge (1983b) reinvestigated the fine structure of *Glenodinium foliaceum,* a "binucleate" dinoflagellate, and found that all plastids and functioning mitochondria of the host-symbiont complex belonged to the symbiont and that the host mitochondria were inactive; on the other hand, the Golgi apparatus, the trichocysts, the starch and lipid reserves, and all microtubules belonged to the dinoflagellate cell. He found that the symbiont of *Glenodinium* was a diatom devoid of a shell.

The mode of division of symbiont nuclei was not well understood since it has been described as amitotic. However, more recently condensation of chromosomes has been described in the symbiont nucleus of *Glenodinium* (Chen and Zhou 1985).

Only about a third of the cells of *Gymnodinium acidotum* retain a nucleus in the endosymbiont which is, in this case, a cryptomonad. The other cells harbour only anucleate symbionts which, however, retain their nucleomorphs. In either case, it is the chloroplasts and mitochondria of the symbiont that are conserved and functional in the symbiotic complex (Farmer and Roberts 1990). In *Peridinium quinquecorne,* all chloroplasts also belong to the symbiont (a chrysomonad), which is separated from the host cytoplasm by a single membrane; the symbiont also retains its mitochondria, Golgi bodies and an eukaryotic nucleus (Horiguchi and Pienaar 1991).

PHYLOGENETIC IMPLICATIONS

In the sixties and seventies, the mesokaryote concept of dinoflagellate organization, first put forward by Dodge (1965), clearly predominated (for review see, e.g., Raikov 1982). This concept postulated that there were not two but three levels of organization of living cells: prokaryotic, mesokaryotic, and eukaryotic. The mesokaryotic level was thought to be characterized by a mixture of prokaryotic

and eukaryotic features. To the former were counted the peculiar highly ordered structure of the dinoflagellate chromosomes comparable with that of certain bacterial nucleoids, the absence of histones and nucleosomes, the allegedly membranous mechanism of mitosis, and the allegedly continuous replication of DNA (absence of a definite cell cycle). Among the latter were: presence of a nucleus limited by a normal nuclear envelope and containing a nucleolus, generally eukaryotic organization of the cell (presence of cell organelles, etc.).

More recent studies showed however that mitosis in dinoflagellates was not purely membranous but involved activity of microtubules. In fact, dinoflagellate mitosis turned out to be a variant of closed extranuclear pleuromitosis comparable to that in metamonads. Also a definite cell cycle was discovered in the dinoflagellates, and repetitive DNA sequences were found to be abundant in their genome, which are further eukaryotic characters. Therefore the number of prokaryotic features of dinoflagellates decreased and the number of eukaryotic features increased. However, it was still possible to consider the dinoflagellates as a sister group to all eukaryotes, that is to admit that they were primitive and had ancient origin (Herzog et al. 1984). This view was in principle compatible with the mesokaryote concept though it placed the dinoflagellates nearer to the eukaryotes than to the prokaryotes. It also stressed some peculiar features of the dinoflagellates, e.g. massive replacement of thymine by 5-hydroxymethyluracil.

The first molecular evolution results obtained with the technique of rRNA sequencing, when the number of organisms compared was still small (and specifically, when ciliates and apicomplexans were not included) seemed to be compatible with the idea that dinoflagellates emerged early, were primitive, and could be a sister group to all (other) eukaryotes, regardless how they were called, mesokaryotes or eukaryotes. This has been maintained after sequencing the 5.8 S rRNA of *Prorocentrum* by Maroteaux et al. (1985) and after sequencing the 17 S rRNA of the same organism by Herzog and Maroteaux (1986). However, most other molecular evolution data (see above) using rRNA sequence comparison show that dinoflagellates cluster with ciliates and apicomplexans, all the three emerging rather late in evolution, and thus cannot be primitive eukaryotes or mesokaryotes. This relationship between dinoflagellates, ciliates, and apicomplexans has already been noticed using approaches of comparative morphology and all three considered to be a monophyletic group named Parvkingdom Alveolata (Cavalier-Smith 1993).

Summing up, the "prokaryotic" features of the dinoflagellates are now reduced to the organization of their chromosomes: highly ordered structure, absence of histones and absence of nucleosomes. All three features are linked together since absence of histones may cause the other two. In fact, formation of nucleosomes clearly depends on the presence of histones and the fact that concentrated pure DNA can crystallize into liquid crystals of ordered structure which resemble dinoflagellate chromosomes (Livolant 1984) also brings the peculiar dinoflagellate chromosome structure back to the lack of histones. All these facts support the idea (Sigee 1986, Rizzo 1991) that dinoflagellates are true, and not even primitive, eukaryotes and that loss of histones in them is secondary, bringing about both lack of eukaryotic chromatin structure (nucleosomes) and the highly ordered structure of condensed chromosomes. The similarity of dinoflagellate chromosomes and prokaryotic nucleoids is then a pure convergence. The loss of histones by the dinoflagellates may be brought about by deletion, in the course of evolution, of the histone genes typical of the eukaryotes (Rizzo 1991). Then, presence of histones, as in *Oxyrrhis,* may be a primitive rather than derived character, which is consistent with placing *Oxyrrhis* into the subphylum (or class) Protalveolat(e)a, more ancestral than "true" dinoflagellates which remain in the subphylum (or class) Dinoflagellat(e)a (Cavalier-Smith 1993, Corliss 1994).

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Development of Ventral Primordia in Pervasive Ciliary Hypertrophy Mutants (PCH) of the Hypotrich Ciliate *Paraurostyla weissei*

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Summary. We studied the development of oral and ventral cirral primordia in double homozygous *mlm/pl* recessive mutant line 20 and its "granddaughter" line 106 expressing pervasive ciliary hypertrophy. The initial location and basal body source for developing oral primordium and fronto-ventro-transverse primordia (FVT) is the same as in wild type (wt) cells except that their number is higher and variable. The number of FVT streaks in the mutants are 136-180 percent higher than in wt, the maximal number of streaks reached 20, whereas 7-8 streaks is the standard for wt cells. In spite of severe overduplication of basal bodies in the FVT streaks the diversification into specific kinds of cirri corresponds fairly well to the wt pattern. We conclude that the two genes *mlm* and *pl* are involved in controlling the extent of basal body proliferation in wt cells, and that the site of action of at least the *mlm* gene includes an event in the cell cycle related to termination of the first wave of basal body duplication.

Key words. Ciliate pattern formation- *mlm/pl* mutants-*Paraurostyla weissei.*

INTRODUCTION

Studies using a genetic approach in dissecting pattern formation have provided a potentially important perspective on control of development not only in multicellular organisms, like *Drosophila* and *Caenorhabditis*, but also in unicellular eukaryotes (Frankel 1992, Beisson 1994). Ciliates have been extensively studied especially with respect to coordinate effects of gene mutations on spatial organization of the cortical cytoskeleton related to basal bodies (Review in Jerka-Dziadosz and Beisson 1990; Frankel et al. 1993; Jerka-Dziadosz et al. 1992, 1995). Among mutants previously described are multi-left-marginal (*mlm*) and pattern lability (*pl*) unique mutations of the hypotrich ciliate *Paraurostyla weissei* (Jerka-Dziadosz and Dubielecka 1985, Dubielecka and Jerka-Dziadosz 1989, Jerka-Dziadosz 1989).

In the course of genetic analysis aimed at defining the genetic background of a particular segregant *mlm* line 95 (Jerka-Dziadosz et al. 1989) new abnormal phenotypes were isolated, all controlled by the two recessive genes *mlm*⁻ and *pl* ⁻. The phenotypes of the double homozygotes included differences in the multiplication of almost all ventral and dorsal ciliary structures, as well as disorders in the adoral membranelles. In the earlier study, five sister progeny lines from one cross expressing the recessive alleles of *mlm* and *pl* genes were analyzed with respect to the number of all categories of ventral structures in non-dividing cells. These lines (18, 32, 77, 56 and 20) differed significantly and formed a series of progressively stronger

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phenotypes, where the expression of the deviation from normal ciliary pattern was gradually amplified.

Analysis of developmental processes in single gene *mlm* mutants had established that a hypertrophy of ciliary structures is caused by specific modifications of morphogenetic processes occurring in an affected cortical domain, the oral and left-latero-dorsal domain (Dubielecka and Jerka-Dziadosz 1989, Jerka-Dziadosz and Wiernicka 1992).

Here we present studies on modifications of development of oral and ventral (FVT) primordia in two *mlm/pl* lines: the severely-affected line 20 and its even more abnormal "granddaughter", line 106. These abnormalities are considerably more severe than those of the previously described *mlm/pl* line 95 (Jerka-Dziadosz, 1989). Nonetheless, we observe that in spite of a variable overduplication of basal bodies in almost all categories of primordia, the spatio-temporal sequence of differentiation, which is specific for each cortical domain, remains approximately normal.

The analysis of abnormalities in these two *mlm/pl* lines and comparison of the previously studied line 95 revealed that the two genes are involved in controlling the extent of basal body proliferation in wt cells, and that the controlling system is highly dynamic and is probably influenced by the genetic background. The action of the *mlm* gene may include the critical event in the cell cycle related to termination of the first wave of basal body duplication, and also may influence the stability of positional values in a morphogenetic field.

MATERIALS AND METHODS

Strains and growth conditions

The following lines of *Paraurostyla weissei* (European strain, Wirnsberger et al. 1985) were used in this investigation: LuV, a wild-type (wt) progeny line from a cross between line Lu collected from fresh water pond in Poland (Lubien), and line Ver, a wt collected in France by Dr. A. Fleury, and other progeny lines from different crosses. Cells were maintained in Petri dishes in Pringsheim solution (Jerka-Dziadosz and Frankel 1969) and fed daily with green alga *Chlorogonium*, grown on SMC medium (Weischer et al. 1985) in an alternating light-dark regime.

Observations on developing primordial structures were performed on dividing cells, reorganizing cells and regenerating cell fragments. The morphogenetic events during regeneration and physiological reorganization follow the same path as developing primordia during cell division, except that one set of primordia is formed rather than two (Jerka-Dziadosz and Frankel 1969).

Cytological preparations

For visualization of the cortical ciliary structures, the silver proteinate (Protargol, Merck) staining method as described previously (Jerka-Dziadosz 1985) was used. Three methods of cell permeabilization and processing were used for immunolabeling of cortical cytoskeletal structures. Procedure 1 is the same as described earlier (Jerka-Dziadosz 1990). Briefly, cells were permeabilized in PHEM buffer (Schliwa and van Blerkom 1981) containing 0.5-1% saponin, 3% bovine serum albumin (BSA) and 0.1% Tween-20, and incubated for one hour in the primary antibody. The cells were then washed twice in buffer without saponin and incubated in the secondary antibody diluted in the same buffer (FITC-labeled goat anti-rabbit IgG antibody, of rhodamine-labeled goat anti-mouse IgG antibody). The polyclonal antibodies were diluted 1:400. After incubation cells were washed twice in PHEM, BSA, Tween-20 buffer and mounted in Citifluor (London).

In the second procedure, cells were permeabilized as above, then washed briefly in Tris-buffered saline (TBS: 150 mM NaCl in 20 mM Tris-HCl, pH 8.2) containing 1-3% BSA, 0.1% Tween-20, 2 mM $MgCl_2$ and 1 mM EGTA and incubated in antibodies diluted in the above buffer.

In the third procedure cells after permeabilization were fixed for one hour in freshly prepared 2% paraformaldehyde in PHEM buffer, then they were washed for 30 min in TBS-Tween, $MgCl_2$, EGTA buffer, next briefly soaked in the same buffer containing additionally 3% BSA, and further processed as in the preceding procedure.

For double labeling with two antibodies, two monoclonal antibodies (mAbs) or a mAb and a polyclonal antibody were added simultaneously to the incubation buffer. Samples incubated with a mAb and a polyclonal antibody were exposed to a mixture of FITC-conjugated anti-mouse and rhodamine-conjugated anti-rabbit antibody.

Samples of 20-30 cells were handled individually in depression slides with micropipette.

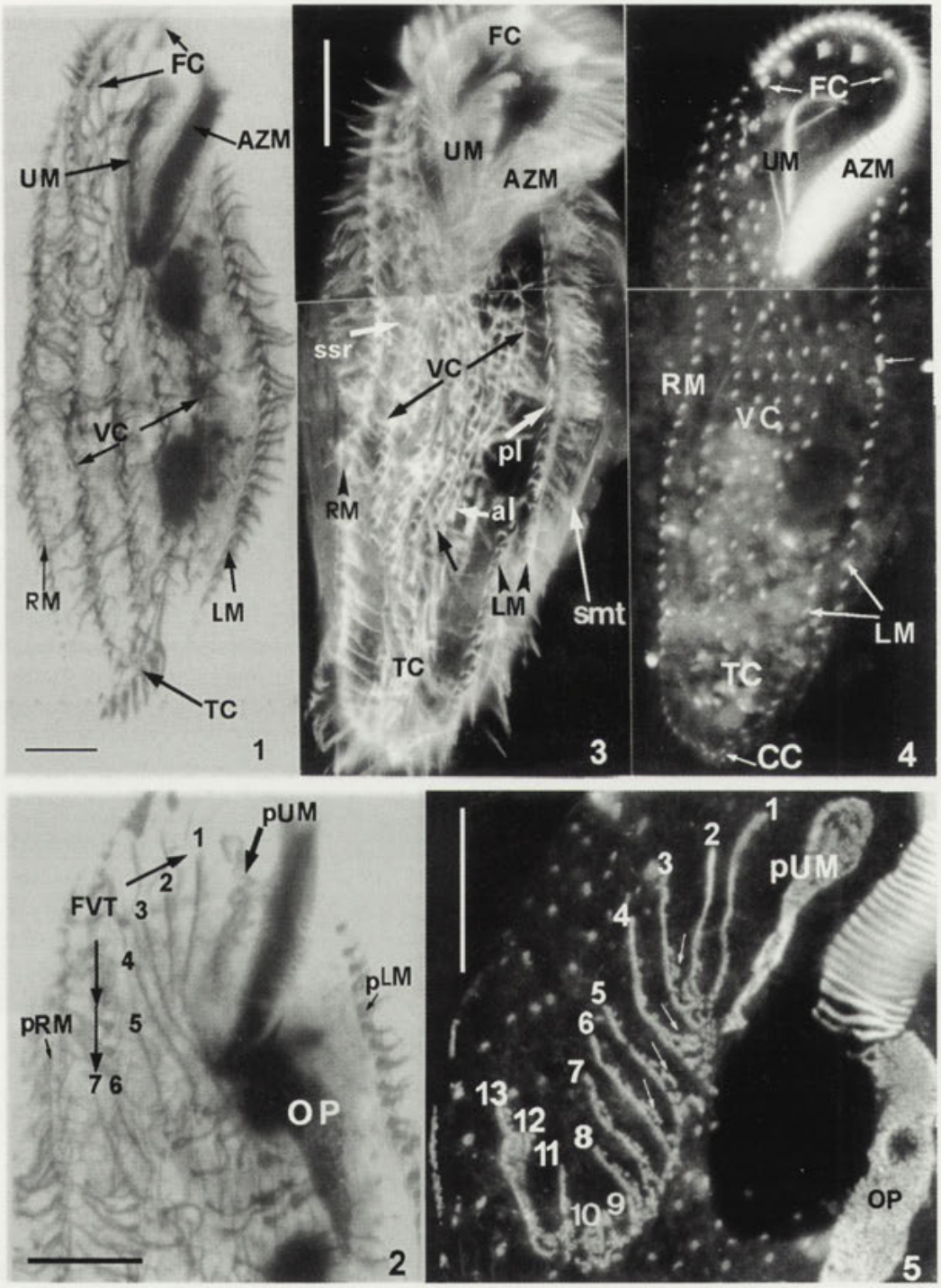
Antibodies: two polyclonal antibodies were used. One, anti-centrin serum 26/14-1 (Erabolu et al. 1994) was a gift from Dr. J. L. Salisbury. The other was an anti-*Paramecium* striated-rootlet antibody (Sperling et al. 1991) kindly provided by Dr. J. Beisson and Dr. L. Sperling. In *Paraurostyla* this antibody was shown to label the striated ciliary rootlets (Jerka-Dziadosz 1990)

The following mAbs were used: first, CC310, a gift from Dr. C. Klotz. CC310 decorates pericentriolar material and striated rootlets in ciliated epithelia of quail oviduct (Klotz et al. 1986); in *Paraurostyla* it labels the bases of cirri and oral membranelles (Jerka-Dziadosz 1990). The second mAb, 10B10, was obtained by Dr. B. Vigues against isolated cortices of *Tetrahymena*. In *P. weissei* it labels the bases of oral ciliature, cirri and dorsal bristles in interfission cells (Jerka-Dziadosz, unpublished study). The antibodies FXI 15D3 and FV10D12 were obtained by Dr. M. E. Nelsen against *Tetrahymena* cortical preparations (Nelsen et al. 1994, Jerka-Dziadosz et al. 1995), In *P. weissei* they label cortical microtubular structures.

The wild-type (wt) cortical pattern

Here we review aspects of the cortical pattern of wt cells and main points of development of primordia of the ciliary structures that are essential for understanding the *mlm/pl* phenotype.

P. weissei is a dorso-ventrally flattened 160-200 μm long hypotrich ciliate with the oral apparatus and the locomotory cirri located on the ventral surface (Fig. 1). The dorsal surface is covered by 5-7 rows of bristle units (DB). The oral ciliature surrounding the endocytic apparatus consists of an adoral zone of membranelles (AZM) and two paroral membranelles called collectively the undulating membrane (UM). The cirri consist of four groups: large frontal cirri (FC) arranged in two oblique rows posterior to the distal part of the AZM, 4-5



Figs. 1-5. The ventral surface of *P. weissei*. Bar - 10 μm. 1 - wild-type interfission cell stained with Protargol. FC - frontal cirri from the anterior group, AZM - zone of adoral membranelles, VC - ventral rows of cirri, LM - single row of the left marginal cirri, RM - single row of the right marginal cirri, TC - transverse cirri, UM - undulating membrane. 2 - wild type cell in stage 3 of physiological reorganization: OP - field of basal bodies forming the primordium of new adoral membranelles; pUM - reorganizing undulating membranes; 1-7 primordial streaks of frontal, ventral and transverse primordia (FVT), pLM - primordium of the left marginal cirral rows, pRM - primordium of the right marginal cirral row. 3-4 - the ventral surface of the PCH 106 interfission cell doubly labelled with monoclonal antibody FV10D12 (Fig. 3) and polyclonal anti-centrin antibodies (Fig. 4). 3 - shows cortical microtubular structures: cilia, cirral rootlets (al - anterior longitudinal fiber, pl - posterior longitudinal fiber, ssr - small subectoplasmic rootlet) and the submembrane microtubular sheath (smt). The basal bodies in cirri are not labelled (arrow). This cell possess eight ventral cirral rows (VC), two left marginal cirral rows (LM) and one right marginal row. 4 - shows the bases of cirri and membranelles. Note the irregular size of some cirri (small arrows). 5 - The FVT primordia in PCH 106 cell labeled with anti-centrin. Numbers 1-13 indicate anterior parts of the streaks. Note the supernumerary streaks formed at the posterior-left parts of the streaks (arrows). Due to compression, the OP is slightly misplaced toward (viewers) right side

longitudinal rows of ventral cirri (VC) occupying most of the ventral surface bordered posteriorly by an oblique row of large transverse cirri (TC). Single longitudinal rows of marginal cirri are located at both the left (LM) and right (RM) margins of the cell. Several caudal cirri (CC) occupy the posterior tip. The number of basal bodies in a cirrus varied in different categories from 8-16 in marginal and ventral cirri, up to 120 in some frontal cirri (Bakowska 1980, 1981).

The cortical development of *P. weissei* is complex, involving the replacement or reorganization of every element of the ciliature except for a part of the adoral membranelles in the anterior division product. These processes have been extensively studied and described in earlier papers (Jerka-Dziadosz 1980, 1981a, b, 1982; Wirnsberger et al. 1985; Fleury et al. 1993).

On the ventral side three major sets of ciliary primordia develop (Fig. 2): first, the oral primordia consisting the adoral (OP) and paroral (pUM) parts which originally form as anarchic fields of basal bodies and subsequently align into specific oral pairs (AZM pairs and UM pairs) and differentiate into new adult structures. Second, the ventral cirral primordia (FVT), and third the primordia of the left and right marginal cirri (pLM and pRM) developing as primordial streaks of paired basal bodies that originate from specific parental cirri (Fig. 2). An ultrastructural aspect of these processes is summarized in recent paper (Jerka-Dziadosz and Wiernicka 1992).

Although there is some variability in the number of structures in interfission cells directly related to the size of cells (Jerka-Dziadosz 1976; Jerka-Dziadosz and Golińska 1977; Bakowska 1980, 1981), in a well fed, growing population the number of ventral cirral structures is fairly stable as is the number of primordial FVT streaks (Jerka-Dziadosz and Frankel 1969, Wirnsberger et al. 1985).

RESULTS

Origin of the double homozygotes *mlm/pl*

The original strain of *P. weissei* showing the *mlm* phenotype was isolated by inbreeding of cell clones collected from fresh water ponds in Poland (Jerka-Dziadosz and Banaczyk 1983). Further study revealed that the phenotype is under control of a single recessive gene at locus termed *mlm*, showing Mendelian inheritance (Jerka-Dziadosz and Dubielecka 1985). Among F2 progenies of clones heterozygous at the *mlm* locus we obtained lines expressing other pervasive pattern modifications in addition to the multiplication of the left marginal cirral rows. It was subsequently documented that this phenotype is controlled by two non-allelic genes: *mlm*$^-$ and *pl*$^-$ (Jerka-Dziadosz et al. 1989).

One of the *mlm/pl* double homozygous clone 20 was then crossed to the wt clone LuV. The heterozygotes phenotypically wt were grown until sexual maturity, and then back-crossed to the parental line 20. Among progeny of this cross, line 4 expressed a *pl*$^-$ phenotype, including occasional duplication of the left marginal cirral row and a single macronucleus; line 31, heterozygous at both *mlm* and *pl* loci, was phenotypically wt. From a cross between lines 4 and 31 we obtained, among other phenotypes, a clone (line 106) expressing the pervasive ciliary hypertrophy (PCH) known from previous study to be controlled jointly by genes *mlm*$^-$ and *pl*$^-$.

Cortical pattern in pervasive ciliary hypertrophy (PCH) mutants

The PCH mutant clones can be easily identified among wt ones by the great variability in size and shape of cells, in particular by increased width of dividing and reorganizing cells.

The main feature of the cortical pattern of PCH cells is the multiplication of practically all ventral and dorsal structures with a slight increase in length (Table 1). On average they possess six or seven frontal cirri (four is normal) of irregular size (Fig. 4) in the first oblique row posterior to the AZM. The FC No. 1 (anterior, left-most), in wt being the largest, in these mutants often is the smallest and is mislocated toward the cell's left side and frequently is misoriented. The number of transverse cirri varies between 10 and 27 (7-9 in wt). Some cirri are

Table 1

Ventral ciliary pattern in non-dividing cells of wt and PCH mutants

		Number of cirri		Number of cirral rows		
Line symbol Pheno-type	Length Range **Mean** SD %CV n	FC Range **Mean** SD %CV n	TC Range **Mean** SD %CV n	VC Range **Mean** SD %CV n	LM Range **Mean** SD %CV n	RM Range **Mean** SD %C n
18a*	125-273	4	7-10	4-6	1	1
	156.24	**4.00**	**8.44**	**5.08**	**1**	**1**
wt	11.10	0	0.71	0.40	0	0
	7.10	0	8.41	7.87	0	0
	25	25	25	25	25	25
20*	121-190	4-11	10-16	6-14	1-4	1-3
	159.32	**6.00**	**13.14**	**9.00**	**2.47**	**2.09**
PCH	16.96	2.80	1.49	1.61	0.67	0.62
	10.64	46.70	11.30	17.80	27.10	29.60
	20	20	20	20	20	20
106	146-194	6-9	15-27	7-11	1-2	1-2
	169.16	**7.25**	**20.65**	**8.95**	**1.90**	**1.25**
PCH	15.24	0.71	3.70	1.25	0.30	0.44
	9.00	9.79	17.91	13.96	15.78	35.20
	13	20	20	20	20	20

* Data from Jerka-Dziadosz et al 1989.
PCH - pervasive ciliary hypertrophy; wt - wild type

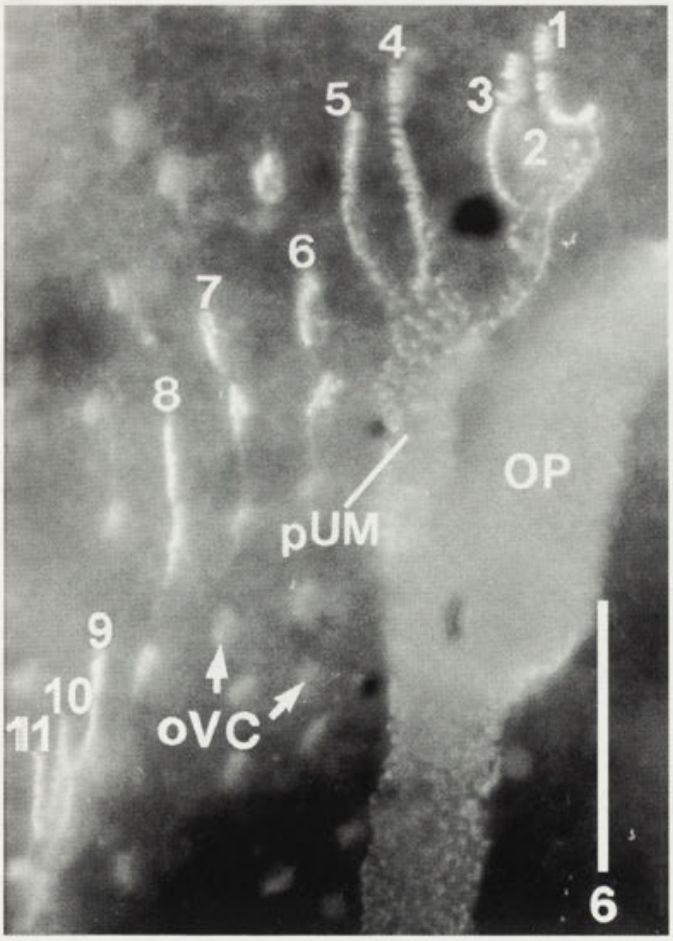
unusually large, as if they originated by fusion of two small ones, others are smaller than normal.

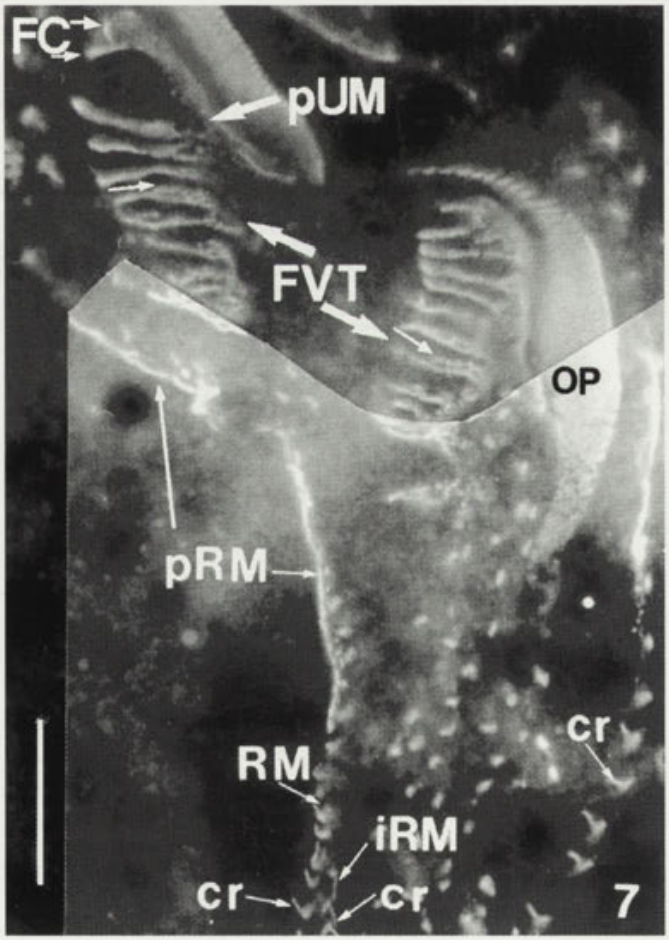
The ventral cirral rows are frequently duplicated. Although their general pattern is similar to wt, that is the left-most rows are the shortest and the middle and right ones are the longest, short rows are often intercalated between long ones in the posterior half of the cell (Fig. 4). An increased number of cirral rows is also found in the marginal (Table 1) and dorsal bristle rows (data not shown).

Protargol and anti-tubulin staining revealed that the whole cell surface in the PCH cells is underlain by the microtubular sheath (Fig. 3) as in wt cells. All cirri, regardless of their size and shape, possess the normal complement of microtubular rootlets: anterior longitudinal fibers (al), posterior fibers (pl) and the oblique subectoplasmic ribbon (ssr). Immunolabeling of the kinetodesmal fiber (striated ciliary rootlet - cr) revealed that all left marginal cirri possess one big fiber. On the right side, the marginal cirri located in the posterior one third of the cell also possess the kinetodesmal fiber. In normal cirral rows these fibers are directed anterior right in inverted rows they point posterior-left (Fig. 7). The dynamics of kinetodesmal fibers during division has been described previously (Jerka-Dziadosz 1990).

Oral primordia in PCH cells

The anarchic field of basal bodies for the primordium of adoral membranelles is frequently hypertrophied both in dividing and reorganizing PCH cells. It appears wider than in wt cells especially in its posterior part, and often extends toward the left side as in other *mlm* lines (Dubielecka and Jerka-Dziadosz 1989, Jerka-Dziadosz 1989). However, contrary to its predecessors, in PCH mutants the differentiation of promembranelles is

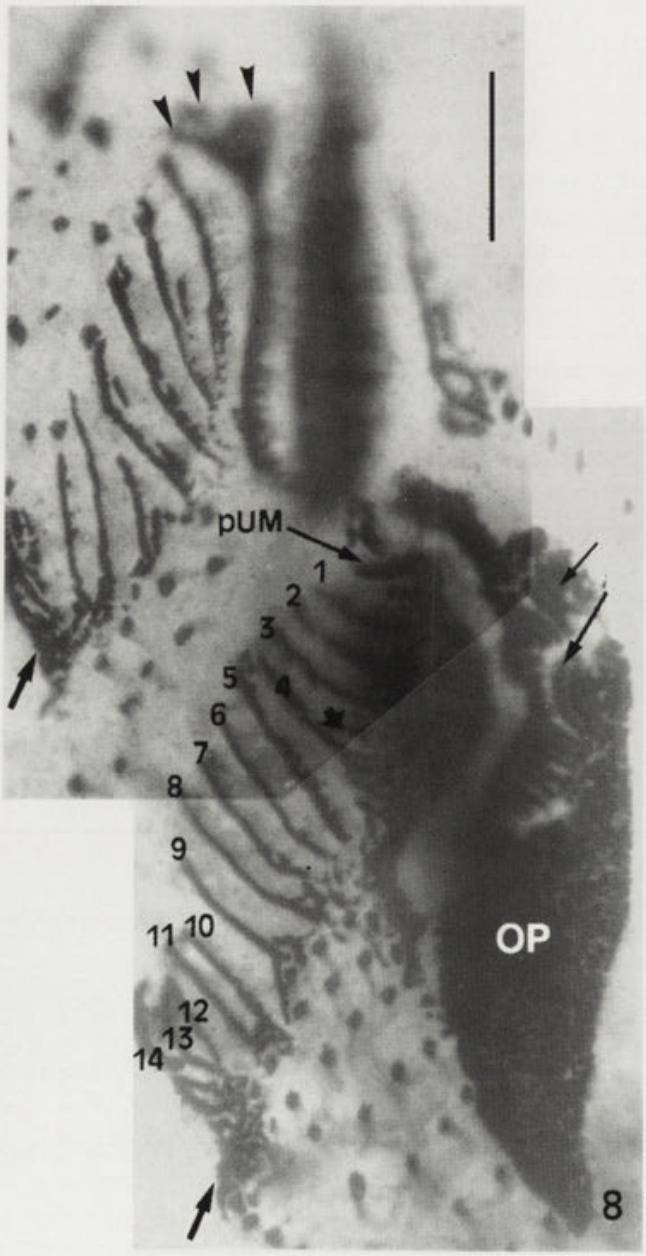


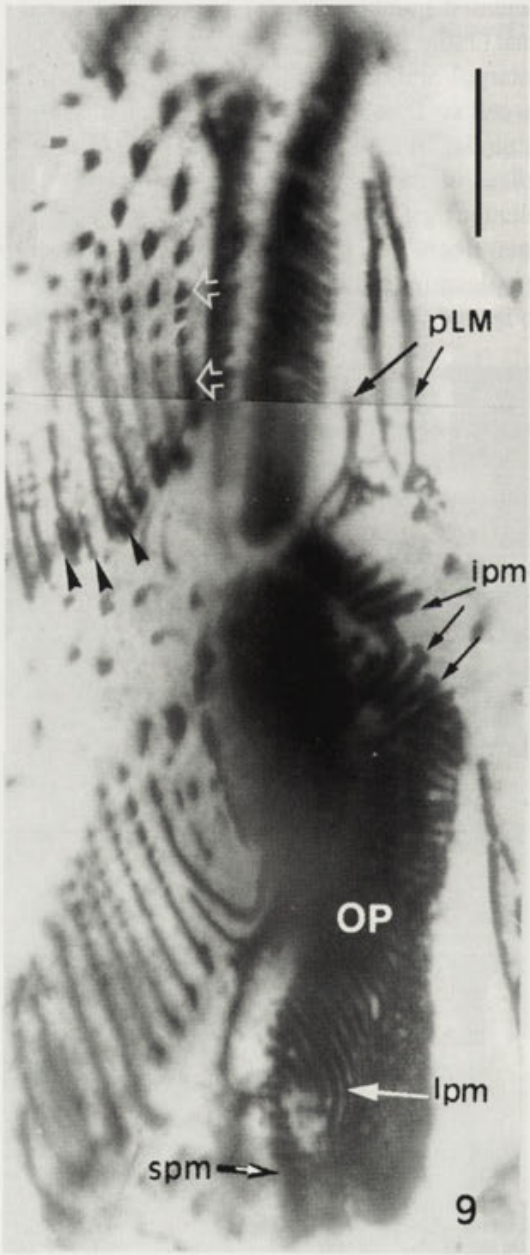


Figs. 6-7. The ventral surface of PCH 106 cells labeled with anti-cr polyclonal antibody. A high concentration (1:200) of the serum permitted visualization of both basal bodies and striated ciliary rootlets (cr). Bar - 10 µm. 6 - the posterior division product at stage 2-3 of division. Note the FVT streaks No. 1-5 abutting the pUM, single streaks No. 6-11 forming within old ventral cirral (oVC) rows. 7 - a dividing cell at early stage 4 of division. Two FC differentiate within the anterior pUM (short arrows). Supernumerary streaks develop on the left side of the longer ones (small arrows). The mother cell possessed two RM rows, the inner one showing antero-posteriorly reversed orientation (iRM) with cr directed posterior-left

modified when compared to wt cells (Jerka-Dziadosz 1981a). In the oral primordium the promembranelles often show modified size and bizarre shape (Figs. 8, 9) and orientation. Some of these abnormally patterned promembranelles are resorbed during the final stages of morphogenesis, although abnormal AZMs persist in about 40% of interphase cells of both PCH lines.

The hypertrophy also affects the primordium of the paroral membranes. Contrary to Wirnsberger et al.(1985) and Fleury et al. (1993) we do not consider the pUM as the FVT streak No.1 (compare Figs. 2, 5) especially since the orientation of basal body pairs and the mode of differentiation of paroral membranes differs from that of FVT streaks (Jerka-Dziadosz and Wiernicka





Figs. 8-9. Dividing cells of PCH 106 cells stained with Protargol. Bar - 10 µm. 8 - the ventral side of a cell in early stage 4 of division. Three frontal cirri (arrowheads) differentiate in the anterior pUM. Note the highly irregular primordium of the AZM (OP) in the posterior division product, arrows point to some of the misoriented promembranelles. The number of FVT streaks in the posterior division product is higher than in the anterior one. The multiplication of streaks is particularly evident in the sector (1-4) adjacent to pUM (thick arrow) and in the right-most sector (arrows). 9 - the ventral side of a cell in late stage 4 of division. New cirri differentiate within the FVT streaks. In the anterior product of division note the differentiation of ventral cirri in streak No. 1 (white arrows) and differentiation of entire short streaks into TC (arrowheads). The primordia of the left marginal cirri are highly hypertrophied (pLM). The posterior AZM possesses irregularly long (lpm), or short (spm) promembranelles in its posterior part and inverted (ipm) in the anterior part

1992). The pUM field appears thicker in PCH mutants than in wt, and up to four frontal cirri differentiate on its anterior right side. These cirral primordia are clearly visible on cells labelled with the anti-cr antibody (compare Fig. 7 and also Fig. 16 in Jerka-Dziadosz 1990).

FVT ciliary primordia in PCH mutants

The developmental phenotype of PCH line 95, described earlier (Jerka-Dziadosz 1989) included hypertrophy of the oral, left marginal and left dorsal primordia, as well as hypertrophy of right marginal and right dorsal primordia, whereas the FVT primordia in the majority of developing cells were nearly normal or slightly overgrown (Jerka-Dziadosz 1989). However, the increased number of frontal, ventral and transverse cirri in other PCH lines (Jerka-Dziadosz et al. 1989) suggested severe abnormality in their development as well, and therefore led to this study of development in the more severely affected PCH lines 20 and 106.

Analysis of early stages of development of the FVT streaks in these clones revealed: first, the relative location of the initial sites of basal body proliferation along the antero-posterior axis of the cell is the same as in wt cells. Second, the majority of the streaks develop in duplicate form, expanding laterally.

In the anterior division product and in reorganizing cells, the first two streaks originate within the FC of the second row posterior (as in wt) to the distal part of the AZM (Figs. 5, 6), and the next one is formed either from the third FC or slightly posterior, depending on the configuration of the preexisting cirri. The next three to six streaks (two in wt) are formed with participation of some old ventral cirri, located to the right of the ventral part of the old AZM, whereas the right-most group of FVT streaks is formed within the right most ventral cirral row. This part of the FVT, in wt cells consisting of two overlapping segments initially formed within one old cirral row (Wirnsberger et al. 1985), is very severely hypertrophied in PCH mutants (Figs. 5, 8) and often consists of up to five parallel segments.

In the posterior division products (opisthes) the origin of the first FVT streaks differs from that in the anterior ones. The initial location and basal body source for developing FVT streaks is the same as in wt cells, except that their number is higher and variable. Usually there are 3-4 streaks connected with the longitudinal field of basal bodies located adjacent and parallel (Fig. 6) to the oral anlagen (pUM), 2-3 streaks formed within the ventral cirral rows and up to 5 streaks formed within the right-most ventral row (Figs. 7, 8). As in the anterior division products, the streaks are irregular and often of abnormal length, and the spacing between the streaks is non-uniform. In some dividing cells forming very high number of FVT streaks, these streaks are slanted toward the pUM with their posterior ends (Fig. 7).

In early stage 3 of developing wild type cells, all 7 or 8 streaks are composed of basal body pairs aligned into a longitudinal row with well delineated left and right sides (Fig. 2). Single, or paired basal bodies are occasionally visible near the posterior ends of streaks supplying basal body pairs until late stage 4.

In sharp contrast to wt, in PCH cells of both studied lines, in almost all cells the streaks are usually well delineated (on Protargol preparations and immunolabelled cells) only on the right side (Figs. 5, 7). This corresponds to the posterior basal body of each pair, which is equipped with the striated ciliary rootlet (Jerka-Dziadosz 1980, 1990). On the left side of the streaks loose basal bodies, basal body pairs, short segments of streaks or small fields of dispersed basal bodies are visible (Fig. 5, small arrows), resulting from an overproliferation of basal bodies. Some of the supernumerary streaks are resorbed, others undergo the second round of basal body proliferation, that is they thicken and later differentiate into new cirri (Figs. 8, 9), as do the primary streaks.

In order to compare quantitatively the developmental phenotype of the PCH mutants with wt pattern, we analyzed the development of the abnormal FVT sets in two PCH lines: *mlm/pl* 20 and its "granddaughter" *mlm/pl* 106. The number of primordial streaks was counted in cells in early stage 4 (Fig. 8) of divisional morphogenesis in the anterior and posterior products of cell division as well as in cells engaged in physiological reorganization, in which one set of primordia is formed. It can be noted, first, that in all lines presented in Table 2 the posterior division products develop more primordial streaks than anterior ones, observations consistent with an earlier study on wt cells (Jerka-Dziadosz 1976). Second, the mean number of streaks in PCH lines 20 and 106 shows a 136-180 percent increase compared to wt cells with the number being higher in line 106 than in line 20. The maximal number of streaks reached 20, whereas 7-8 streaks is the standard for wt cells. Third, the number of FVT streaks is highly variable.

A matrix of old TC and new FVT streaks in posterior division products of dividing wt and PCH 106 cells in stage 4 is shown in Table 3. In wt cells, about 40% of cells produced the same number of FVT streaks as they had produced in the previous generation (closely ap-

Table 2

Developmental phenotype in wt and PCH mutants

Line symbol Pheno-type	Proter Range **Mean** SD %CV n	Opisthe Range **Mean** SD %CV n	P+O+R Range **Mean** SD %CV n	FC Range **Mean** SD %CV n	TC Range **Mean** SD %CV n
		FVT primordia		Preexisting cirri	
LuV	6-8	6-9	6-9	4	7-9
	7.00	**7.54**	**7.27**	**4**	**7.63**
wt	0.77	0.82	0.82	0	0.67
	11.00	11.87	11.27	0	8.78
	11	11	22	11	11
20*	8-11	8-13	8-13	4-6	10-15
	9.50	**10.87**	**10.23**	**4.87**	**12.25**
PCH	0.92	1.64	1.43	0.99	1.66
	9.60	15.08	13.97	20.32	13.55
	8	8	17	8	8
106	9-15	11-19	9-19	4-9	12-24
	12.63	**14.81**	**13.53**	**6.33**	**16.33**
PCH	1.91	2.44	2.43	1.49	4.28
	26.20	16.47	16.99	23.53	26.20
	11	11	22	15	15

* Data from Jerka-Dziadosz et al 1989.
PCH - pervasive ciliary hypertrophy; wt - wild type

proximated by the number of old TC). In sharp contrast, in PCH 106 cells only 10% of the cells produced the same number of streaks as they produced TC in the previous morphogenetic process. It appears that each dividing cell is different with respect to the relation between the number of FVT streaks and old TC, indicating a lack of coordination between the number of formed FVT streaks with the same structures formed in previous morphogenesis.

Differentiation of new cirri

In PCH mutants, the anterior frontal cirri differentiate in the anterior-left side of the pUM and in FVT streaks 1-4. The posterior frontal cirri differentiate within the first three left FVT streaks. The transverse cirri are formed at posterior ends of all FVT streaks. The middle part of the FVT streaks segments into ventral cirri (Fig. 9). This general pattern of diversification into specific kinds of cirri corresponds fairly well to the wt pattern. The modes of the striated ciliary rootlet differentiation characteristic for each category of cirri are the same as in wt cells and have been described previously (Jerka-Dziadosz 1990).

The differences between wt and PCH cells are quantitative rather than qualitative. For example: (1) both Wirnsberger et al. (1985) and Fleury et al. (1993) documented for the European wt strains that the single FC No. 1 originates from the pUM (compare Fig. 8 in Fleury et al. 1993). In PCH mutants, up to five FC (usually 2-3) may differentiate in the anterior part of the pUM. In the first two FVT streaks of wt cells, two frontal and one transverse cirrus differentiate, whereas in PCH cells in addition to that several ventral cirri develop from the middle part of the streak (Fig. 9, white arrows). This indicates that there not only is an overall multiplication of the streaks, but within some streaks an increased number of basal body pairs undergo the second wave of basal body proliferation giving rise to supernumerary ventral cirri. (2) The short, secondary streaks located in the posterior part of the FVT (Figs. 8, 9, arrowheads) often are entirely transformed into single large transverse cirri- a situation never happening in wt cells where each TC has its corresponding frontal and/or ventral cirri derived from the same streak.

The morphogenetic movements of differentiating new cirri generally correspond to those in wt cells. Modifications are found in some frontal cirri, especially the supernumerary FC formed in the anterior part of the

Table 3

Relationship between the number of preexisting TC and the number of FVT streaks in opisthes from dividing cells at stage 4

wild type (line LuV and 10)

TC	6	7	8	9	
FVT	Number of cells				Total
5		1			1
6	1	3	1	1	6
7		4	4		8
8		2	3		5
9		1			1
Total	1	11	8	1	21

Line 106

TC	12	14	15	16	17	18	21	22	23	24	
FVT	Number of cells										Total
9	1				1						2
10	1			1							2
11					1	1					2
12			1								1
13								2			2
14					1					1	2
15			1	1			1		1		4
16										1	1
17		1			1						2
18				1							1
19		1									1
20							1				1
Total	2	2	2	3	4	1	2	2	1	2	21

pUM. These new cirri are displaced left toward the AZM, and often remain more posterior and do not connect to the microtubular system accompanying the distal part of the AZM (Bakowska and Jerka-Dziadosz 1978). These FC often display modified orientation, and some of them may be resorbed at final stages of morphogenesis and/or during interphase.

The termination of a morphogenetic process involves resorption of the remnants of old ciliature and of those new basal bodies which were assembled too late to be incorporated into the new structures. In PCH mutants, these processes are modified only quantitatively. All old ciliary structures become disassembled and replaced by new ones. The timing of this resorption corresponds to that in wt cells. Some of the newly formed "secondary" structures are also resorbed, usually those which were formed relatively late. Part of the expanded adoral primordium which does not differentiate into membranelles also becomes resorbed.

DISCUSSION

In the series of mutants: from *pl* expressing the single recessive allele at the *pl* locus, through *mlm* mutants expressing the single recessive allele at the *mlm* locus, the double *(mlm^-/pl^-)* recessive PCH mutants of *P. weissei* display the most severely modified ciliary pattern (Jerka-Dziadosz et al. 1989). The common characteristics of these mutants are overduplication of basal bodies and variability in expression of the modified phenotype. The various abnormalities found in these clonal lines are not found in every cell, nor are they equally pronounced in all cells. This allows three tentative conclusions: first that the two genes mlm^+ and pl^+ are involved in controlling the extent of basal body proliferation in wt cells; second, that the controlling system is multigenic as it is influenced by the genic background within a given ciliate strain, and third that it is highly dynamic.

Control of basal body proliferation

In search of the likely cause of basal body overduplication in PCH mutants we should consider two possibilities: structural abnormality of basal bodies and abnormal timing of the morphogenetic process. First, in developing cells the basal bodies themselves, the peribasal material as well as the microtubular rootlets and striated fibers associated with them appear ultrastructurally normal (Jerka-Dziadosz and Wiernicka 1992) and display the same immunolabeling pattern as wt cells by anti-centrin, anti-tubulins: FXI 15D3, FLIII 2C7 and FVLIV 12G10, anti-cr, CC310 and FV 10D12. Abnormality in the basal body or in its associated structures is highly improbable although not impossible since molecular mechanisms of basal body assembly are largely unknown, and the immunological probes used are far from exhausting the richness of structural components of basal bodies and their immediate surrounding (Kalt and Schliwa 1993, Archer and Solomon 1994).

Centrin mutants of the flagellate *Chlamydomonas* express increased and variable number of flagella with seemingly normal basal bodies (Taillon et al. 1992). The primary defects are apparently very different in three mutants of *Paramecium* expressing overduplication (*kin 241, cro1*) or underduplication (*sm19*) of basal bodies (Ruiz et al. 1987, Jerka-Dziadosz et al. 1992, Jerka-Dziadosz and Beisson 1993). Thus it appears that modifications in the quantity of basal bodies are brought about by diverse mutated events occurring upstream or downstream of the basal body assembly.

The initiation and termination of the assembly of basal bodies are tightly controlled events in the cell cycle. In the hypotrich ciliates assembly of new basal bodies is restricted to a defined part of the cell cycle. It begins at early prophase of the micronucleus (Mi) and the terminal part of the S phase of the macronucleus. The association between basal body assembly and the mitotic cycle of the Mi is constant in all developmental situations (Jerka-Dziadosz and Frankel 1969, Jerka-Dziadosz and Janus 1975), whereas the macronuclear cycle could be dissociated from the cortical development (Jerka-Dziadosz and Frankel 1970). The first wave of basal body proliferation, which is responsible for the number of new ciliary elements, ends by mid-metaphase in wt cells, the second wave defining the size of new ciliary structures ends around late telophase. Assembly of new basal bodies was not found in interphase cells.

As concerns the timing of morphogenetic processes, the overduplication of basal bodies in PCH cells does not result from premature initiation of basal body assembly. The beginning of a morphogenetic process in *P. weissei* could be marked by means of the Vigues's 10B10 Mab (Frontczak and Jerka-Dziadosz 1990) which in interphase cells binds to the peribasal body material in cirri and membranelles as well as to the cirral baskets (Jerka-Dziadosz, unpublished EM study). At the onset of basal body assembly in the anarchic field, the 10B10 antigen disappears from old structures and reappears in differentiating new structures at terminal stages of

development. There is the same coordination of the break down of the 10B10 labeled cytoskeletal elements with the beginning of the assembly of new basal bodies in wt and PCH mutant cells (data not shown).

Previous analysis of timing of basal body proliferation in *mlm* mutants revealed that the first wave of basal body assembly continues beyond the point of termination in wt cells (Dubielecka and Jerka-Dziadosz 1989). This may indicate that the site of action, at least of the *mlm* gene, includes a critical event in the cell cycle related to termination of the first wave of basal body duplication.

PCH phenotype and genic background

In previous studies on modification of ciliary pattern in non-dividing cells from six sister clonal PCH lines (Jerka-Dziadosz et al. 1989), it was established that they all express variant states of the pervasive multiplication of ciliary structures. Morphometric and statistical analysis showed that the extent of overduplication of particular groups of ciliary structures differed significantly in each sister line. Similar variability was found also in single gene *mlm* mutant lines (Dubielecka and Jerka-Dziadosz 1989).

Comparison of two PCH lines, 20 and 106, also revealed differences. Cells from line 106 (the "granddaughter" of line 20) on the average possessed more FC and TC, and fewer left and right rows of marginal cirri. In dividing cells of line 106, there are 3-4 more FVT streaks than in line 20, however the number of ventral rows in non-dividing 106 cells is almost the same. This may indicate that in both lines a maximal "crowding" of ventral cirri is reached, and excess developing cirri are resorbed.

The cause of differences in phenotypes of lines 20 and 106 is unknown. It is interesting to note that these differences are less profound than differences between sister *mlm/pl* lines analyzed previously (Jerka-Dziadosz et al. 1989). It could only be speculated that the expression of *mlm*$^{-}$ and *pl*$^{-}$ genes is influenced by other gene products which may either channel the abnormalities into specific meridional regions (e.g. line 95, Jerka-Dziadosz 1989) or cause their spreading onto the whole cell surface. These interactions are probably multistep and indirect, since phenocopies of some aspects of the PCH phenotype occur in the symmetry-reversed component of the mirror-image doublet cells of *P. weissei* (Jerka-Dziadosz 1985).

Dynamics of developmental phenotype in PCH mutants

The developmental phenotype of all PCH mutants is based on two facts: an overduplication of basal bodies resulting in production of enlarged oral primordia and supernumerary primordial streaks, and the lack of fidelity in transmission of ciliary pattern in consecutive cell generations.

It has been previously documented for marginal cirral rows that the number of primordial rows in any morphogenetic process is not directly related to the status of the preexisting ciliature (Jerka-Dziadosz 1989). In FVT primordia in wt cells, the number of FVT streaks corresponds to the number of old TC (Jerka-Dziadosz and Frankel 1969, Wirnsberger et al. 1985). Comparison of the mean number of old TC and FVT streaks in dividing and 106 PCH cells (Table 3) shows an interesting modifications in the relationship of these parameters. The lack of coordination between the number of old TC and the number of developing FVT streaks indicates that in this mutant the number of FVT streaks is not coordinated with the status of the previous generation, but depends on some process taking place during the current morphogenetic event. It is possible that this process includes the target of the genes responsible for the pervasive ciliary hypertrophy phenotype.

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ACTA
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Inhibition of *Leishmania* Growth but not of Phagocytic Functions of Macrophages by 5-fluorouracil *in vitro*

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Summary. We studied the effect of 5-fluorouracil on the growth of two leishmania strains. A concentration of 20 μM inhibited over 95% of the growth of 0.5 x 10^5 promastigotes/ml in three days. Growth inhibition near 50% was observed with 2 μM 5-fluorouracil in three days at cell concentrations ranging from 2.5 x 10^3 to 2.5 x 10^6 cells/ml. A 44 molar excess of thymidine protected by 80% from the effect of 5-fluorouracil, suggesting that leishmania thymidylate synthase is a target for the compound. The most important changes observed by electron microscopy were in the nucleus. 5-fluorouracil had no apparent toxic effect on macrophage phagocytic functions. The respiratory burst of macrophages was identical with or without 5-fluorouracil and their capacity to ingest erythrocytes was unaltered. Microscopically, the leishmania-macrophage interaction *in vitro* showed that with 5-fluorouracil macrophages destroy the protozoan very effectively. In the absence of the drug, leishmanias thrive inside phagocytic vacuoles of macrophages leading to their progressive destruction.

Key words. *Leishmania major, Leishmania mexicana*, 5-fluorouracil, macrophage, growth inhibition, ultrastructure.

INTRODUCTION

Several agents that inhibit the growth of cultured forms of pathogenic leishmanias have been considered as potential drugs for development as treatment of various forms of leishmaniasis. This has been the case for pentavalent antimonials like sodium stibogluconate (Pentostam) or meglumine antimoniate (Glucantime) (Berman et al. 1982), fungicides like amphotericin B (Prata 1963, Sampaio et al. 1971), sinefungin, imidazole and triazole drugs (Maingon et al. 1992), and herbicides like triflurarin (Chan and Fong 1990). The latter agents make use of similarities in metabolism or protein sequences between leishmanias and fungi or plants.

Other drugs have been developed based on differences in purine metabolism between leishmanias and

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humans. Allopurinol (Pfaller and Marr 1974, Marr and Berens 1977, Berens et al. 1980), thiopurinol (Marr et al. 1982), aminopurinol, 3-deazaguanosine, 9-deazainosine, formycin A, formycin B and analogs of guanine, guanosine (LaFon et al. 1985, Avila et al. 1987), and a carbocyclic analog of 2'- deoxyguanosine (Dong et al. 1992), have been tested *in vitro*, in animal models (Berman et al. 1987) and, in some cases, in patients, with variable results (Kager et al. 1981, Jha 1983, Chunge et al. 1985, Saenz et al. 1989, Martínez and Marr 1992).

Little attention has been given to drugs that affect pyrimidine metabolism in leishmanias because it is substantially similar to that in humans (Marr and Berens 1985, Marr 1991). It has been reported that the antimalarial pyrimethamine is moderately effective at higher doses against cutaneous leishmaniasis and that fused pyrimidines and π-deficient pyrimidines are not significantly active as leishmanicidal agents (Ram 1990).

Even though there are many treatments for localized cutaneous or visceral leishmaniasis of low toxicity and relatively high effectiveness, these are not effective for the cure of disseminated cutaneous leishmaniasis. In recent years patients with visceral leishmaniasis resistant to treatment with existing drugs, as well as patients with severe, incurable forms of the disease have increased (Rocha et al. 1980, Bryceson et al. 1985, Mebrahtu et al. 1989, Barral et al. 1991, Kreutzer et al. 1991, Magill et al. 1992). Thus the search for alternative agents for treatment of diverse forms of the disease continues and has increased within the last years (Maingon et al. 1992).

5-fluorouracil (5-FU) is a pyrimidine analog that has been used extensively as an antineoplastic agent systemically as well as topically (Calabresi and Chabner, 1990). It must be metabolically activated to 5-fluoro-2'-deoxyuridine-5'-monophosphate, which blocks thymidylate synthase, or 5-fluorouridine triphosphate, which incorporates into RNA, causing cytotoxicity.

We have found that 5-FU is an excellent inhibitor of growth for leishmanias in culture and that thymidine protects from this effect suggesting that 5-FU blocks the thymidylate synthase of the parasite. It also produces important morphological alterations in the ultrastructure of the nucleus and the cytoplasm of leishmanias. On the other hand, this compound has no effect on viability, respiratory burst or phagocytic capacity of macrophages, allowing them to eliminate the parasite with high efficiency.

MATERIALS AND METHODS

Parasite cultures and incubations with 5-fluorouracil (5-FU)

Leishmania major (a gift of H. Moll, Institute of Clinical Microbiology, University of Erlangen - Nürnberg, Germany) and *L. mexicana* isolate of a patient with diffuse cutaneous leishmaniasis in Mexico (MHOM/MX/85/Solas) (a gift of A. Monroy, Escuela Nacional de Ciencias Biológicas del Instituto Politécnico Nacional, México) (Kreutzer 1990) were grown in RPMI 1640 supplemented with 10% fetal calf serum at 28°C and 5% CO_2. In most experiments 5-FU (Roche, México, DF) was added only once at the beginning of the culture and cells were counted after three days. In some experiments parasites were allowed to grow until the log phase (for 3 days) before the drug was added. Thymidine (Sigma Chemical Co., St. Louis, MO) protection experiments were performed by culturing *L. major* in the presence of 72 μM 5-FU and variable concentrations of the nucleoside.

The changes in ultrastructure produced by 5-FU in *L. major* were observed in promastigotes grown for 48 h in the absence or presence of the compound (1.1 mM). The parasites were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at 4°C, dehydrated and embedded in epoxy resin and cut with a diamond knife; slides were observed in a Jeol electron microscope model JEM-1200 EXII, at 60 kV.

Action of 5-FU on macrophages

Mouse peritoneal macrophages were obtained by a modification of the method described previously (Becker et al. 1988). Briefly, the peritoneal cavity was washed with phosphate buffered saline and the glass adherent population was enriched by incubating the cells with a glass slide inside a Petri dish in RPMI 1640 at 37°C in 5% CO_2 for 1 h. Differential counts always resulted in more than 80% macrophages with more than 90% viability .

Respiratory burst in mouse peritoneal macrophages in the absence or presence of 1.1 mM 5-FU was measured in a luminometer (LKB-Wallac, Finland) using 5 x 10^{-4} M phorbol-12-myristate-13-acetate (Sigma Chemical Co., St. Louis, MO) as inductor and luminol (Eastman-Kodak, Rochester, NY) as substrate (Frankenburg et al. 1992).

The effect of 5-FU on the phagocytic capacity of mouse peritoneal macrophages was measured by incubating purified cells with heat aggregated rabbit gamma globulins for 30 min in RPMI 1640 at 37°C in 5% CO_2. They were washed with medium and incubated in the presence or absence of 1.1 mM 5-FU for 24, 48 or 72 h. At the end of this incubation sheep erythrocytes sensitized with rabbit gamma globulins were added in a proportion of 20 red blood cells per macrophage and incubated for 1 h. The macrophages were washed with RPMI 1640 and uningested erythrocytes were lysed with 1.55 mM ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM EDTA pH 7.4. Cell viability was monitored throughout the experiment by Trypan blue exclusion. The macrophages were fixed in acetone and endogenous peroxidase was revealed by diaminobenzidine and hydrogen peroxide (Bernhard and Avrameas 1971). Erythrocytes were counted after counterstaining with haematoxylin.

Leishmania-macrophage interaction

Mouse peritoneal macrophages were washed with RPMI 1640 and added to promastigotes in a proportion of 10 parasites per macrophage,

incubated for 24, 48 and 72 h in medium with or without 1.1 mM 5-FU at 30°C in 5% CO_2.

Viable and dead intracellular parasites and the viability of macrophages were assessed by fluorescence microscopy with acridine orange and ethidium bromide as described elsewhere (Streck et al. 1987). They were observed by microscopy with Nomarski optics and by confocal microscopy.

RESULTS

5-FU inhibited the growth of *L. major* in a concentration dependent manner (Fig. 1). The growth was inhibited almost completely at concentrations of 8.5 µM or higher.

1.1 mM 5-FU showed no cytotoxicity or inhibition of respiratory burst of macrophages induced by phorbol-12-myristate-13-acetate during 2 h (data not shown). After this time the signal of the respiratory burst diminishes to undetectable levels in the luminometer.

Inhibition of growth by 50 % was produced within 3 days by 2 µM 5-FU for both strains of leishmanias studied (Fig. 2). Similar percentages of inhibition were observed when the initial concentration of cells cultivated with 2 µM 5-FU was in the range of 2.5×10^3 to 2.5×10^6 cells/ml.

To investigate if this compound is cytotoxic for cultures in the log phase of growth, we tested the effect of 5-FU on two strains of leishmanias cultured without drug for 3 days (Fig. 3). The growth of both strains was affected, but *L. mexicana* was initially more susceptible to the compound and did not grow by day 20. *L. major* showed no growth inhibition until day 6 and then diminished during the next 9 days. (The apparent recovery after day 18 is because the control tubes for *L. major* also have very few cells per tube by day 20, and the curve represents a ratio.) Experiments in which 0.5, 1 and 2 mM 5-FU was added daily during 5 days to 2.5×10^5 cells/ml showed no significant differences with controls with only one initial addition (data not shown).

Protection by almost 80% from the effect of 72 µM 5-FU on leishmanias was produced by 3.2 mM thymidine after 3 days (Fig. 4).

It is noteworthy that during the 10 months that leishmanias have been cultured in the presence of 5-FU, we have not observed the development of resistant strains.

Ultrastructural analysis of the action of 1.1 mM 5-FU on *L. major* showed that after 48 h of incubation promastigotes have undergone a series of changes. These are: loss of the nuclear envelope and the perinuclear cistern, chromatin fragmentation, an increase in the finely granular electrondense material near the chromatin, a lower number of electrondense particles in the cytoplasm and the presence of granulations without membrane of a different electrondensity in the cytoplasm (Fig. 5).

The absence of effect of 5-FU on the phagocytic capacity of macrophages is shown in Fig. 6. Most macrophages in the presence or absence of 1.1 mM 5-FU ingest 7 erythrocytes or more, even after 72 h incubation.

The effect of 5-FU on leishmania-macrophage interaction is shown in Fig. 7. Uptake of leishmanias by macrophages occurs within the first minutes in presence or absence of the drug (Fig. 7a). A clear difference is observed after 72 h, where in the absence of 5-FU there are almost no macrophages and numerous leishmanias persist (Fig. 7b). At intermediate stages and at 72 h it can be observed that the macrophages (that did not burst) contain intact leishmanias in their phagocytic vacuoles (up to three leishmanias can be seen inside a macrophage). In the presence of 5-FU, in the same lapse of time, leishmanias disappear and macrophages remain (Fig. 7c). In this case, although macrophages initially have intravacuolar leishmanias (data not shown), they disappear completely within 72 h (Fig. 7c).

Finally, the supravital stain shows that in the absence of 5-FU the leishmanias ingested by macrophages are alive and the macrophage is dead (Fig. 8a) and in the presence of 5-FU the intracellular leishmanias are dead and the macrophages are alive (Fig. 8b).

DISCUSSION

Our results show that 5-FU inhibits the growth of two leishmania strains completely at concentrations of approximately 20 µM and above (Figs. 1, 2) at the initial and in the log phase of growth (Fig. 3). Excess thymidine can protect leishmanias from growth inhibition, suggesting that a target for the action of 5-FU is the thymidylate synthase of the parasite (Fig. 4).

Some of the effects of 5-FU can be detected at the ultrastructural level; for example the disintegration of organelles like the nucleus in which the function of thymidylate synthase plays an important role (Fig. 5).

There are no previous *in vitro* studies of the cytotoxic activity of pyrimidine analogs on leishmanias. In an earlier study, the compounds 5-cyano-6-(4-fluorophenyl)-2-thiouracil, 3-ethyl- 2-ethylthio-6-(4-fluorophenyl)-4-oxopyr-imidine-5-carbonitrile, 3-allyl-2-allylth io-6-(4-fluoro-phenyl) -4-

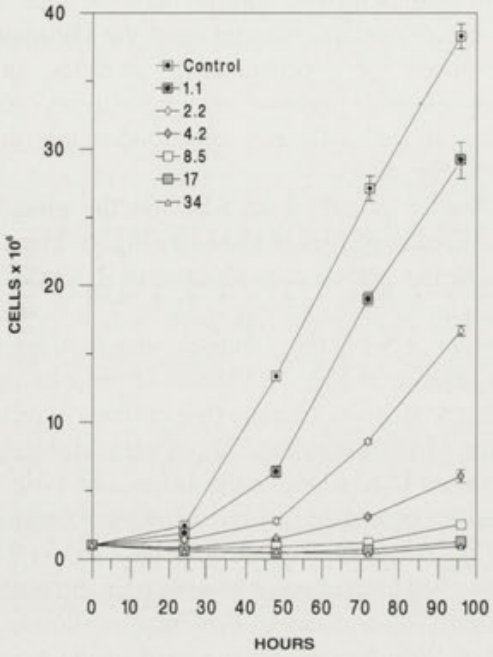


Fig. 1. Growth curves of *L. major* in the absence (Control) and presence of different concentrations (μM) of 5-FU. Promastigotes (1 x 10^6) were cultured in 2 ml tubes in the presence of the indicated micromolar concentrations of 5-FU. Parasites were counted using a hemacytometer after 24, 48, 72 and 96 h, respectively. Each point represents the mean and standard deviation of five tubes

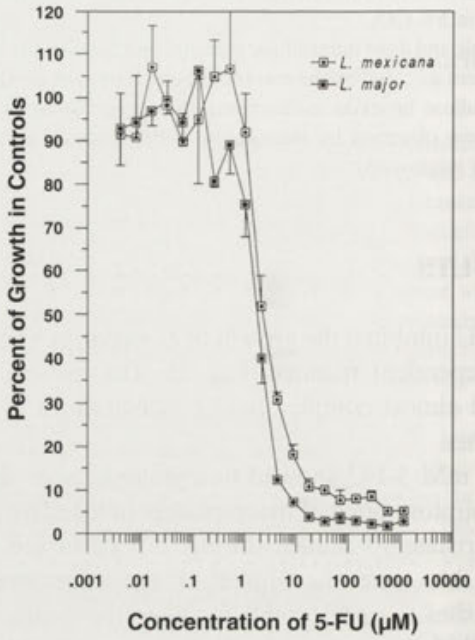


Fig. 2. Representative experiments of the inhibition of growth of *L. major* and *L. mexicana* isolate MHOM/MX/85/Solas by 5-FU. Promastigotes (1 x 10^6) were cultured in 2 ml tubes in the presence of the indicated concentrations of 5-FU. After 3 days, the parasites were counted by hemacytometer and the percentage of growth in the control tubes was calculated. Each point represents the mean and standard deviation of three tubes

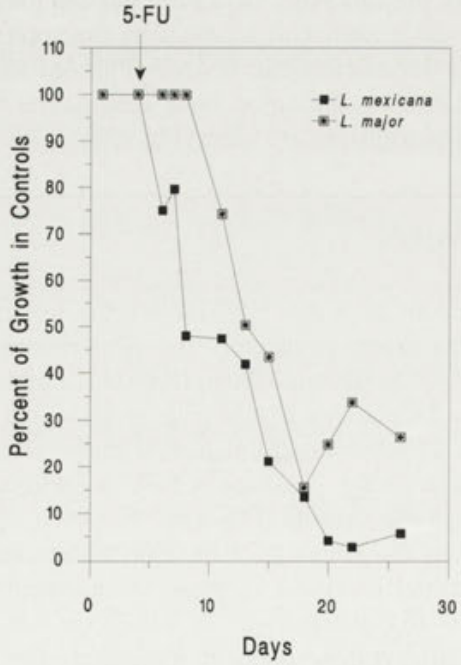


Fig. 3. Representative experiment of the inhibition of growth in the log phase of *L. major* and of *L. mexicana* by 5-FU. Promastigotes (5 x 10^5) were cultured in 2 ml tubes in RPMI 1640. After 3 days, 5-FU was added to a final concentration of 23 mM. The parasites were counted by hemacytometer in the days indicated and the percentage of growth with respect to the control tubes was calculated

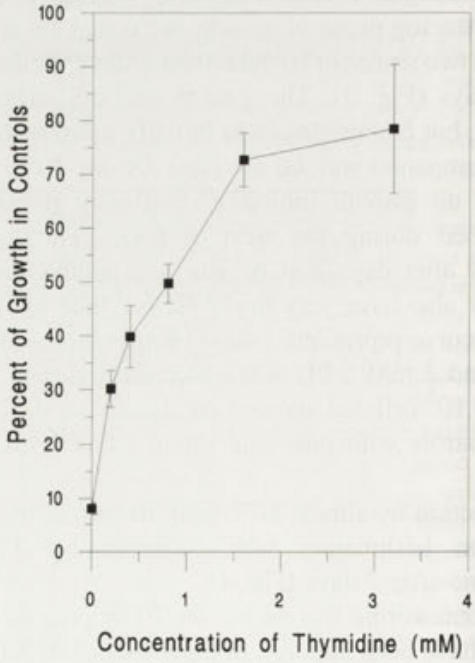


Fig. 4. Reversion by thymidine of the effect of 5-FU on the growth of *L. major*. Promastigotes (5 x 10^5) were cultured in 2 ml tubes in the presence of 72 μM 5-FU and the indicated concentration of thymidine. After 3 days, the parasites were counted by hemacytometer and the percentage of growth in the control tubes was calculated. Each point represents the mean and standard deviation of three tubes

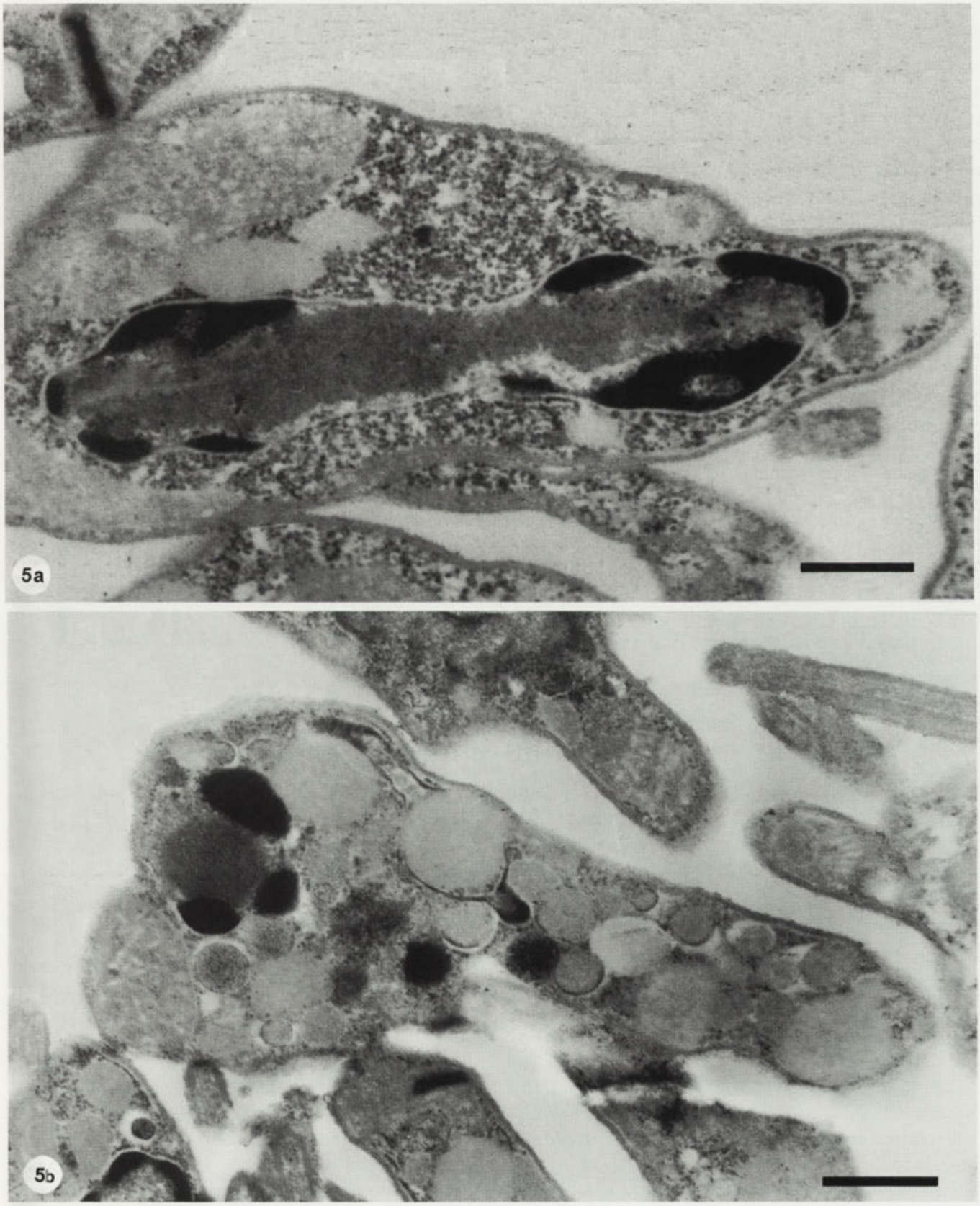


Fig. 5. Ultrastructural changes induced by 5-FU on *L. major* in culture. a - promastigotes cultured without 5-FU, b -promastigotes cultured in the presence of 1.1 mM 5-FU during 48 h. Bars - 500 nm

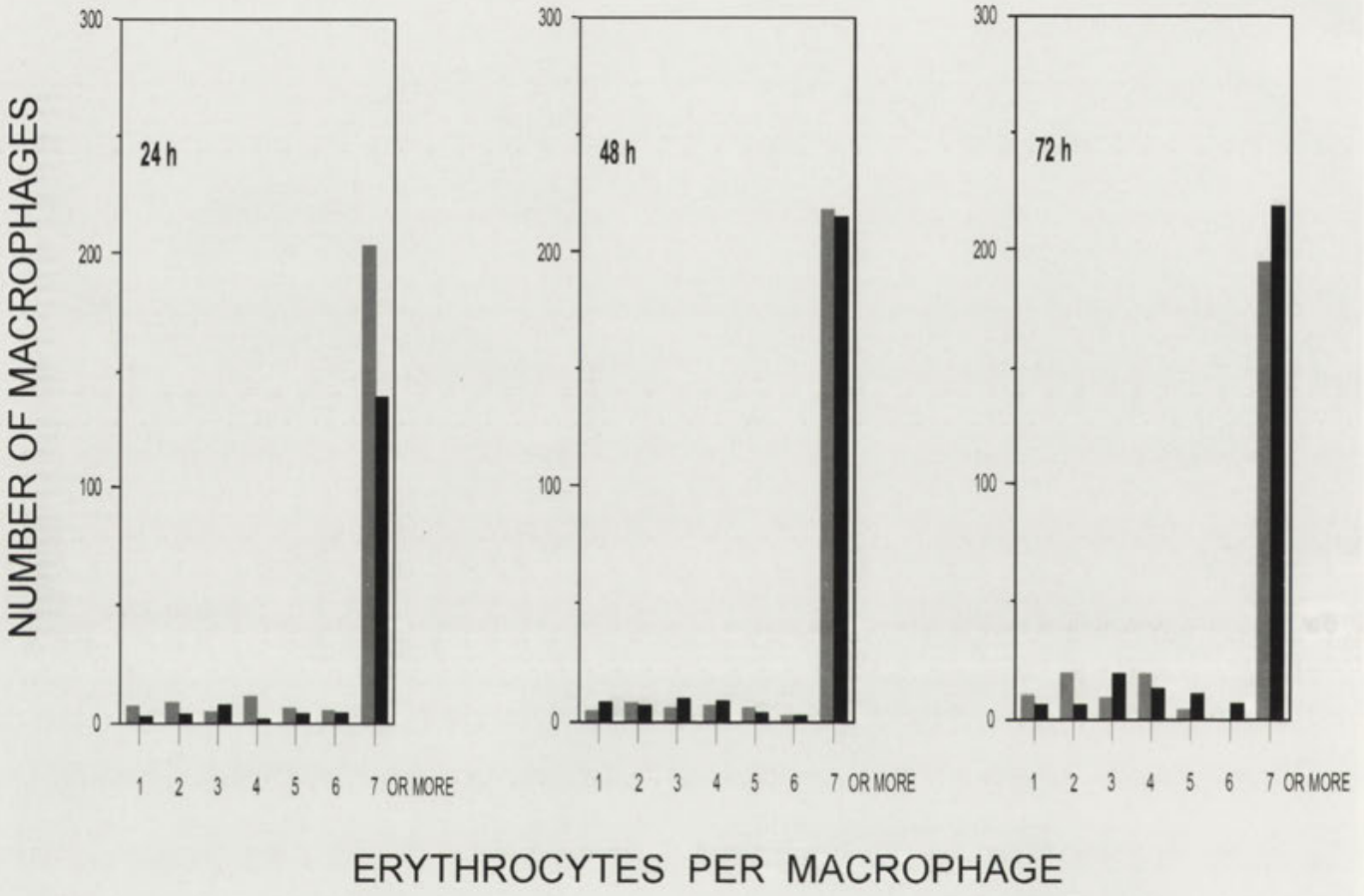
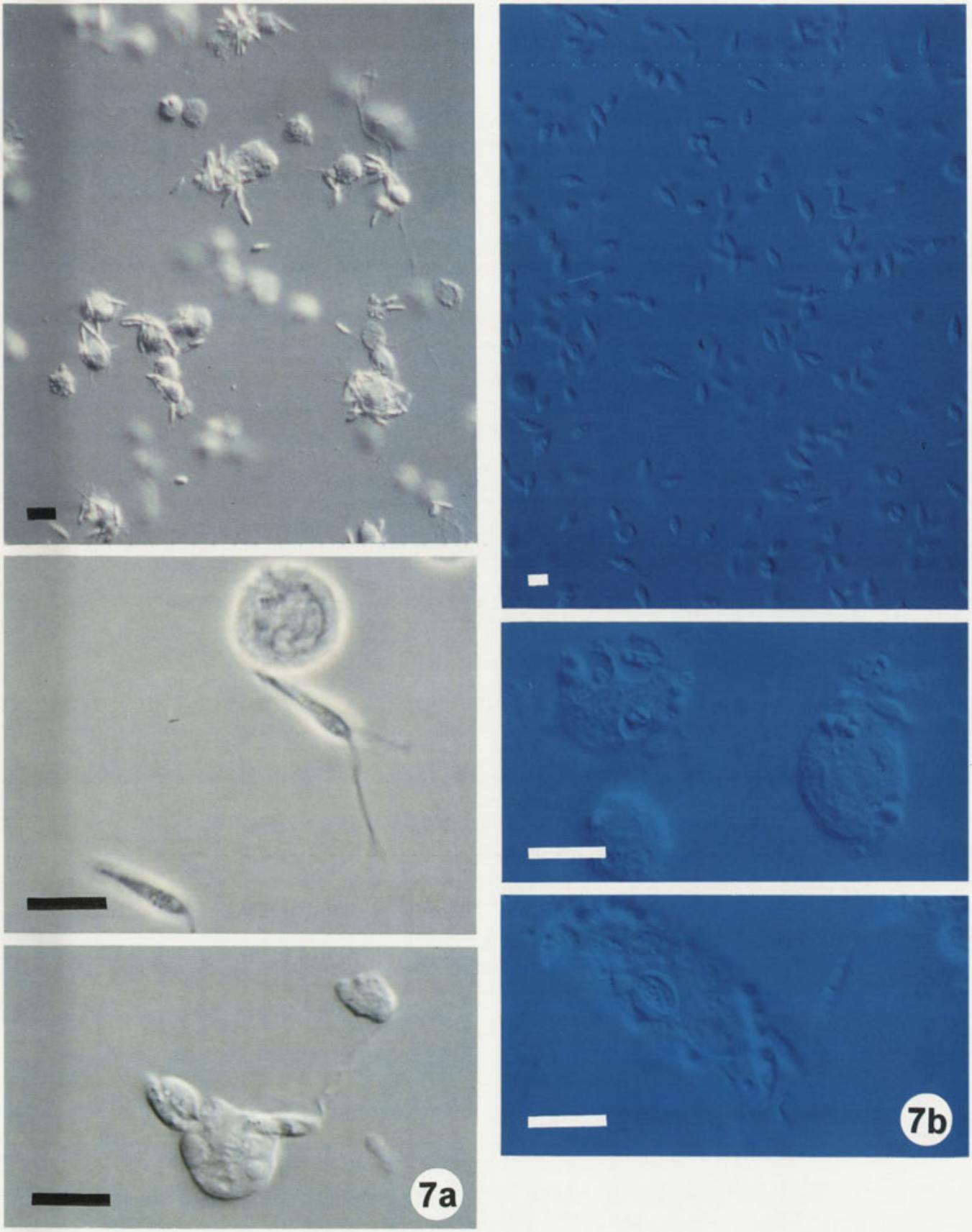


Fig. 6. Effect of 5-FU on the phagocytic capacity of mouse peritoneal macrophages. The gray bars represent control conditions in absence of 5-FU and the black bars represent experimental conditions in the presence of 1.1 mM 5-FU. Note that most macrophages are able to ingest 7 or more erythrocytes at 24, 48 and 72 h

oxopyrimidine-5-carbonitrile,3-benzyl-2-benzylthio-6-(4-fluorophenyl)-4-oxo-pyrimidine-5-carbonitril,2,3-dihdro-7-(4fluorophenyl)-5-oxothiazolo [3,2-a] pyrimidine-6-carbonitrile and 8-(4-fluoropenyl)-6-oxo-2,3,4-trihydropyrimido [2,1-b] thiazine -7-carbonitrile, were tested in hamsters for leishmanicidal activity (Ram 1990). The last compound showed a maximum protection of 28% and this degree of resistance was considered insignificant. These results, together with the experience with pyrimethamine, suggest that extensive substitution of the pyrimidine nucleus may make it less effective as a cytotoxic drug against leishmanias *in vivo*. As could be anticipated, 5-FU does not have a toxic effect on mouse peritoneal macrophages for prolonged periods of time at concentrations 50 times as high as the lethal concentrations for leishmanias, as evidenced by viability, the intact respiratory burst response in the presence of the drug, and the undiminished phagocytic capacity even after 72 h with 5-FU (Fig. 6). Of course, rapidly growing mammalian cells will be affected by 5-FU (hence its use as a chemotherapeutic agent in cancer) but since macrophages are the only cellular hosts for in vivo leishmaniasis, it was important to demonstrate that their phagocytic functions



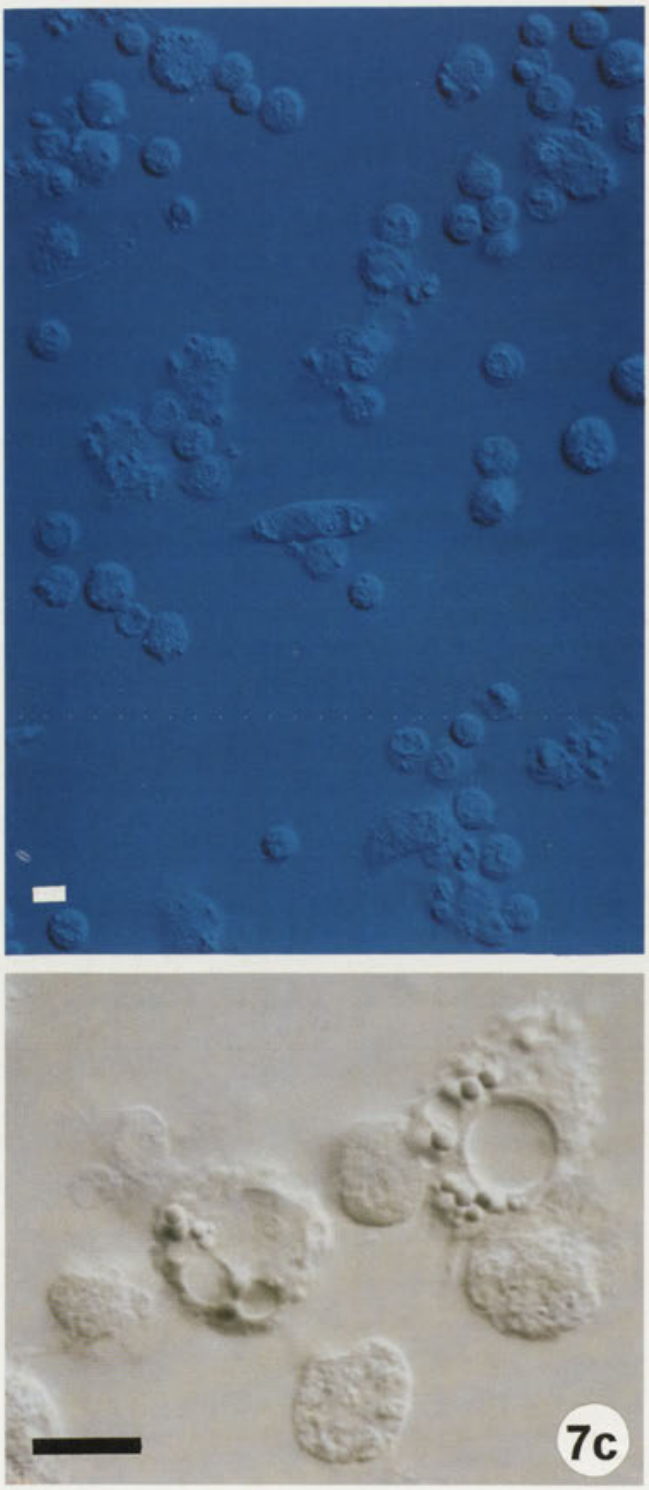


Fig. 7. Effect of 5-FU on the interaction of leishmanias and macrophages. a - in absence of 5-FU, immediately after mixing them. (The image is indistinguishable in the presence of 1.1 mM 5-FU at that same time.) Note in some cases the penetration of macrophages by leishmanias; this occurs during the first minutes of coincubation. b -in absence of 5-FU at 72 h. Note at the lower magnification the absolute predominance of leishmanias and the absence of macrophages; at a higher magnification one can appreciate that the phagocytic vacuoles of the scarce macrophages contain abundant parasites. c - with 1.1 mM 5-FU at 72 h. Note that almost no extracellular or intracellular leishmanias are observed and abundant macrophages remain with empty phagocytic vacuoles. Bars - 10 µm

are not affected at the concentrations used in this work. Yet it cannot be excluded that this base analog could play a role in the reproduction phase of the cell cycle of the macrophage *in vivo*. Our results indicate that 5-FU has an important inhibitory and cytotoxic effect on leishmania *in vitro* that extends over several days. This may be due to the fact that 5-FU is metabolized to toxic and persistent nucleotide derivatives. The effect of 5-FU on the pyrimidine metabolism of leishmania, besides causing important morphological alterations in these cells (Fig. 5), seems to render them more susceptible to killing by macrophages *in vitro* (Figs. 7, 8).

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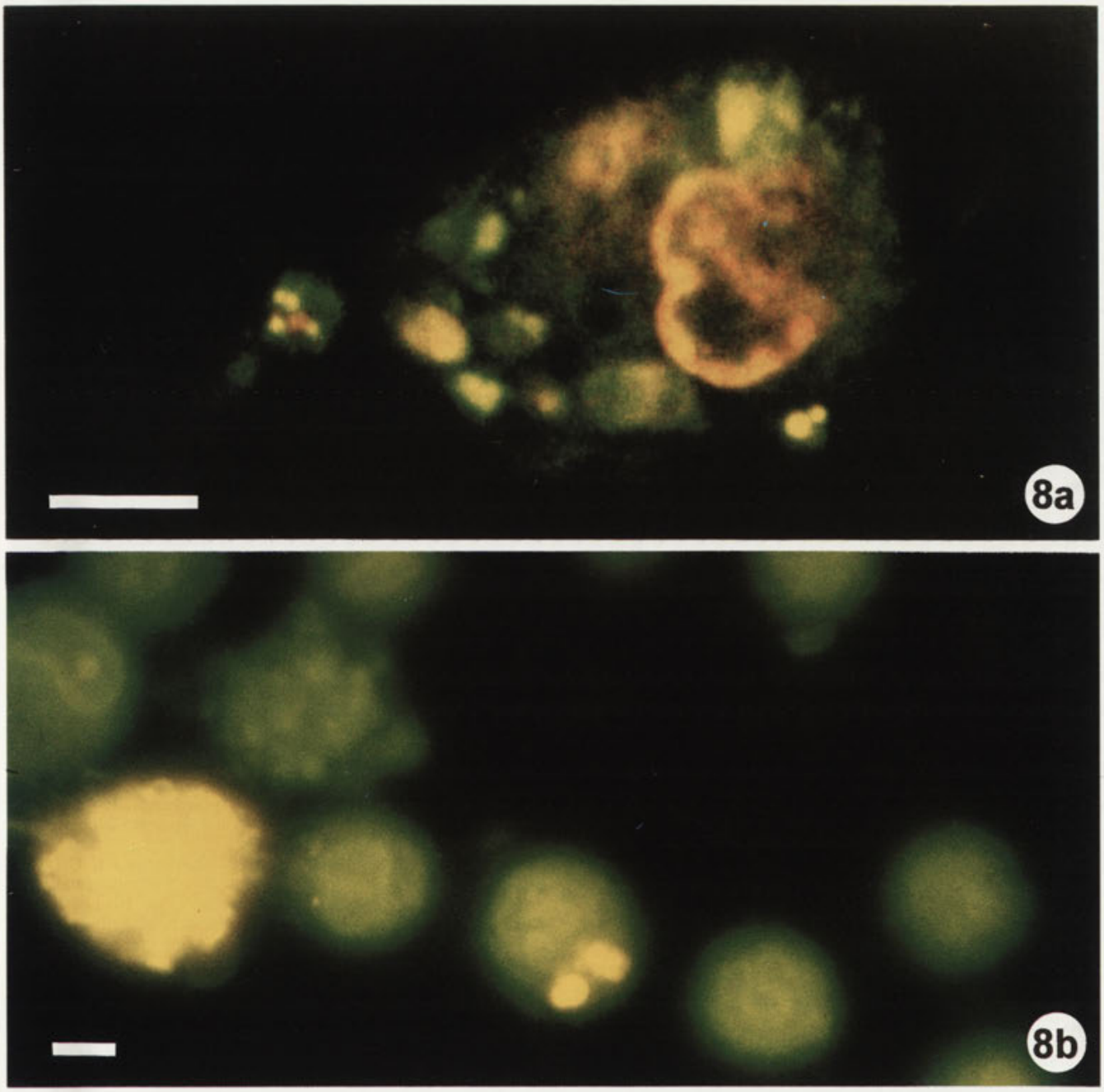

Fig. 8. Effect of 5-FU on the viability of *L. major* in the phagocytic vacuoles of mouse peritoneal macrophages. Promastigotes were coincubated with macrophages during 72 h in the absence or presence of 1.1 mM 5-FU. They were stained afterward with acridine orange and ethidium bromide, fixed and observed in a confocal microscope (a) or in a microscope with epifluorescence (b) with an excitation wavelength of 480-495 nm. The viable intracellular leishmanias, incubated without 5-FU, show a green fluorescence inside a dead macrophage with an orange nucleus (a), and dead parasites or vacuoles of macrophages containing digested leishmanias incubated in the presence of 5-FU show a yellow color (b), whereas the macrophages remain viable showing a green fluorescence. Bars - 10 μm

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Benthic Ciliates of Salt Lakes

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Summary. The number of species and species composition of the benthic ciliate communities have been investigated for 9 salt lakes in Saskatchewan (Canada) and Egypt; these have been compared with one another and with the terrestrial ciliate community of dry salt lakes in the Coorong (Australia). The most important chemical parameters were measured and the bodies of water were classified by Maucha diagrams. The Canadian lakes are athalassohaline, with Mg2+ and SO_4^{2-} as the dominant ions. Lake Qarun in Egypt and the lakes of the Coorong in Australia are thalassohaline, their main ions being Na+ and Cl-. The salinity of the lakes ranges from S= 3.73‰ to 370.2‰. Attention was directed primarily to the infraciliature of all the ciliates detected here so that the species could be identified unequivocally, enabling comparison with populations in other habitats. Altogether, 96 species were identified. The ciliates *Cladotricha edaphoni* sp. n., *Cladotricha halophila* sp. n., *Euplotes pterotae* sp. n. and *Spathidium macrostomum* sp. n. were previously unknown. They were illustrated, described and characterized biometrically. The coenoses can be assigned to three isolated groups corresponding to the different habitats. The species deficit with respect to the athalassohaline lakes reflects the fact that many ciliates differ in their salt tolerance depending on the relative amounts of the various ions.The ciliate coenosis in the dry bottoms of the Coorong lakes is clearly distinct from those of the saline lakes. It is a pure edaphic community comprising 18 species Pomp and Wilbert (1988). When the composition of ciliate coenoses is evaluated from a synecological viewpoint, the existence of differential species becomes apparent. In the present case, the genus Bakuella was found to include such differential species. This study and those of Mihailowitsch (1989) and Mihailowitsch and Wilbert (1990) show that the Bakuella species are predominantly halophilic ciliates. In none of these salt lakes could an interstitial fauna be found. It follows that the lakes studied here originally had a freshwater character Wilbert (1986). Conversely, in the lakes of Siebenbürgen, known to be relicts of seas, ciliates are present in the interstitial fauna Entz (1904).

Key words. Benthic ciliates, taxonomy, ecology, saline lakes, *Cladotricha edaphoni* sp. n., *Cladotricha halophila* sp. n., *Euplotes pterotae* sp. n., *Spathidium macrostomum* sp.n .

1. INTRODUCTION

The sessile organisms living at the bottom of a body of water-communities of bacteria, fungi, protozoa and algae attached to living or inorganic substrate, play a crucial role in transforming the substrate, by breaking down its organic constituents and other activities. They are a significant component of the overall benthic population, and they are of central importance to the energy balance of the body of water (Bick 1964a, Wilbert 1969, Nusch 1970, Bick and Bertram 1973).

Limnological research on the benthos has concentrated chiefly on the usefulness of these organisms as a measure of water quality (Kolkwitz and Marsson 1909, Zelinka and Marvan 1961, Liebmann 1962, Sládecková and Sládecek 1966, Sládecek 1969). Because of this practical value, benthic organisms in general have been thoroughly studied - including the ciliates, the dominant category in number of species as well as individuals. However, very little attention has been paid to the benthos of inland saline waters (Entz 1904, Thienemann 1913, Kahl 1928, Ruinen

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1938, Stiller 1963). To remedy this deficiency, the benthic ciliate fauna of salt lakes in Egypt and Canada has been investigated. Lakes of different concentrations were selected, so that as the populations were being assessed, the influence of the salinity factor on the ciliate fauna could be evaluated.

For the events in a complex biocenosis to be understood, it is necessary to know something about the biology and ecology of the organisms as well as their systematic classifications. The present paper also makes a contribution in this regard.

2. THE BODIES OF WATER INVESTIGATED

In the undrained regions of lakes, both electrolytes (chloride and carbonate of sodium) and nutrient salts (phosphate, nitrogen compounds) accumulate, making these regions saline and eutrophic.

2.1. General characterization of saline waters

Two primary factors affect life in the water: the particular physical characteristics of the water, in its role as the medium in which plants and animals float and locomote, and its chemical constituents, which determine its capacity to provide and distribute the materials that fuel life processes.

In the present context the chemical properties of inland waters are of special interest. The salts dissolved in the water are present in the form of ions. These are chiefly the anions Cl^-, $SO_4 2-$, HCO_3- and $CO_3 2-$, plus the cations K^+, Na^+, Ca^{2+} and Mg^{2+}. Water is distinguished on the basis of the most abundant anion: carbonate, sulfate or chloride (Löffler 1961). In a few regions of the earth there are borate and nitrate lakes. Salt lakes with ionic composition like that of the oceans are called thalassic; those with a different composition are athalassic.

According to Williams (1966), the demarcation point between freshwater and saltwater is a salinity (S) of 3‰ (Bond 1935).

Salt waters are classified on the basis of their salinity, a concept that originated in oceanography and is defined "... as the total amount of dissolved substances contained in a kilogram of seawater under the assumption that all carbonates have been converted to oxides, the bromides and iodides replaced by chlorides and the total organic substance fully oxidized ..." (Remane 1958). Schmitz (1959) defined brackish water as water with a salt concentration between that of pure seawater and that of pure freshwater. As yet there is no generally valid classification of inland saline waters. A thorough discussion of the problems encountered in setting up uniform categories has been published by Hammer (1986).

2.2. The salt lakes in Saskatchewan, Canada (Fig. 1)

The salt lakes of Saskatchewan are situated between the latitudes 51° and 52° N and the longitudes 104° and 110° W . In area, they range from 307 km^2 (Big Quill Lake) to 4.64 km^2 (Waldsea Lake).

The lake basins are glacial in origin; none is over 10 m deep, and they contain water all year round, except for Muskiki Lake, which regularly dries up (Haynes and Hammer 1978). The water in the lakes comes from rain and melting snow. The annual precipitation is 350 mm. For five to six months the lakes are frozen. During the year the water temperature fluctuates around 20°C. Deadmoose Lake and Waldsea Lake are meromictic; the other lakes circulate completely and are stratified in summer (Hammer 1978 a,b,c).

The bodies of water in which the ciliate populations were to be assessed were selected to cover a range of salinities, from a maximum of S = 370.2‰ (Muskiki Lake) down to S = 3.73‰ (Wakow Lake). In Fig. 3 their chemical composition and salinity are represented in ion field diagrams according to Maucha (1932). Na^+, Mg^{2+} and sulfate ions predominate; that is, the lakes

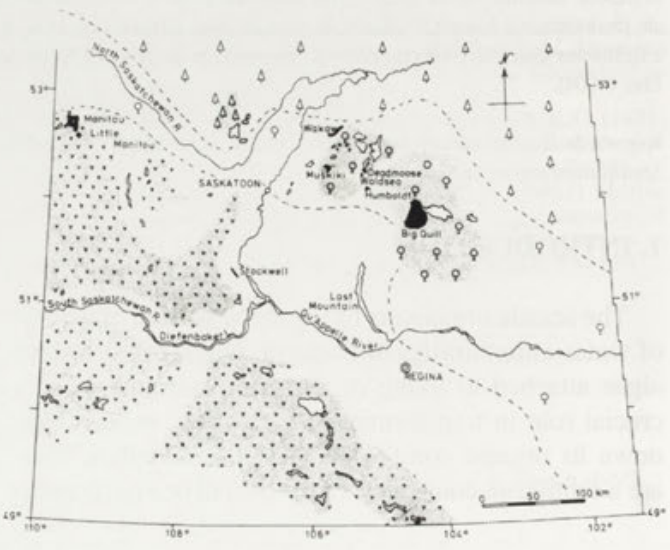

Fig. 1. Map of southern Saskatchewan, showing locations of the lakes in the region under study. Endorheic regions shaded. Modified from Richard and Fung (1969)

are athalassic, with a composition different from that of the ocean.

2.3. Lake Qarun, a salt lake in the Fayum Oasis, Egypt (Fig. 2)

Lake Qarun has an area of about 200 km^2. Its greatest length, from southwest to northeast, is 40 km, and its greatest width is about 5 km. The water temperature ranges from 14°C in January to 32°C in July. The difference in temperature between surface and bottom is no more than 2°C. There is no thermal stratification (Boulos 1959). According to Löffler (1958), Lake Qarun is a tropical lake with frequent general circulation; it is "warm polymictic"

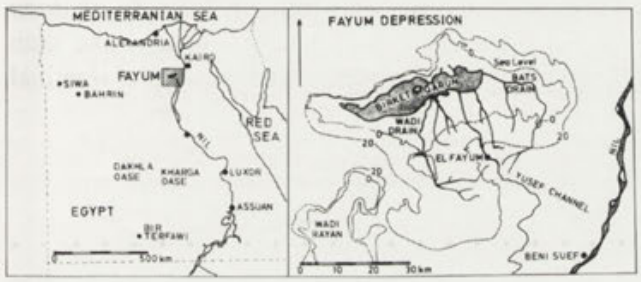

Fig. 2. Lake Qarun, situated in the Fayum Depression

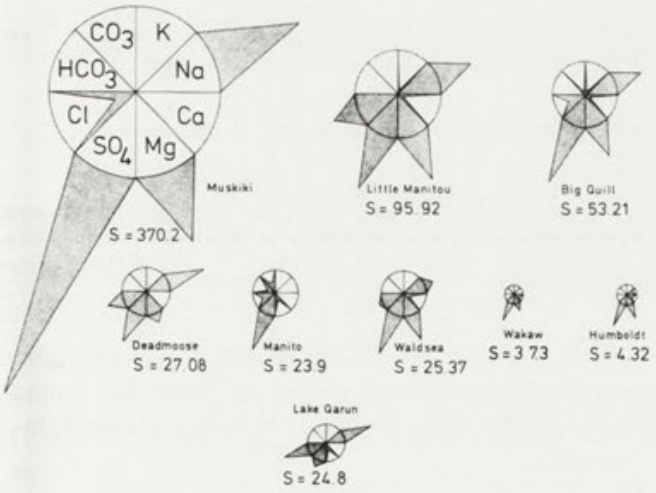

Fig. 3. Salinity and ion field diagrams of the lakes under study. The ionic equivalent percentages of important anions and cations, diagrammed according to Maucha (1932). Scale: diameter of Little Manitou - 2717 VAL

In contrast to the sulfate lakes in Saskatchewan, Lake Qarun is thalassic in character, with sodium and chloride ions dominating (Fig. 3). It is situated in the Fayum Depression, 70 km south of Cairo. According to radiocarbon tests of fossil pollen in the lake sediment, it has existed for 9000 years (Mehringer et al. 1979). In this time the lake was a freshwater lake at an altitude of *ca* 20 m, whereas now it is about 45 m below sea level.

This sinking has created a basin with no drainage that now receives water from twelve streams, all of which were originally tributaries of the Nile. An undrained depression, in the prevailing climate, provided the conditions for salinization of the lake. At present its salinity is somewhat below that of the Mediterranean Sea (Fig. 3).

2.4. The Australian lakes

The saline inland lakes of Australia are temporary, pond-like bodies of water filled with water only in the austral summer from May to August. It is then that the annual precipitation falls, amounting to 500-600 mm/year in southwestern Australia. Precipitation is far exceeded by evaporation of water, over 1400 mm/year. Due to this water loss and the leaching of minerals from the weathering rocks in the surroundings, the salt concentration of the lakes can rise to many times the salinity of the ocean.

The bodies of water relevant here are thalassic saline, with Na^+ and Cl^- as the predominant ions. They are located in the Coorong, a lagoon region created by tectonic shifts in the Pleistocene (Fig. 4).

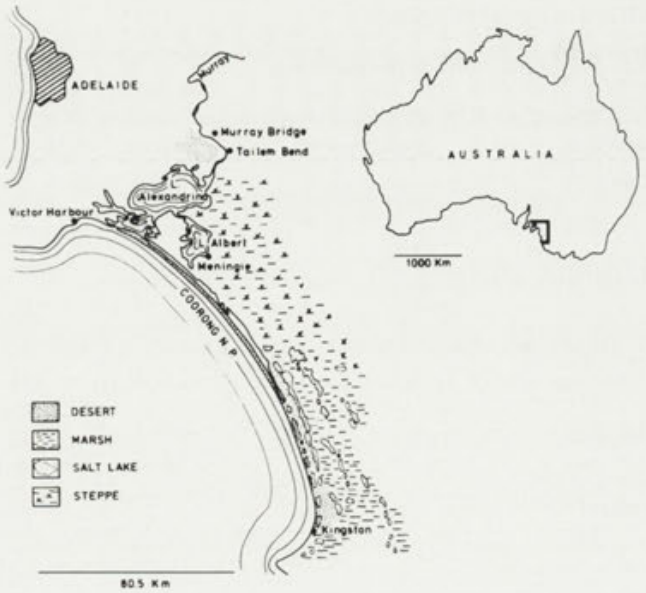

Fig. 4. The salt lakes in the Coorong National Park, from which the soil samples were taken after the lake bottoms had dried up

3. MATERIALS AND METHODS

3.1. Examination of the benthos and soil samples

The material collected in Canada and Egypt came from the upper littoral region of the lakes. In sandy parts of the shore I also investigated the interstitial fauna, using the seawater-ice method of Uhlig (1964, 1968). The ciliates from Australia were collected in samples taken in January 1986 from the dry lakes in the 0- to 10-cm horizon.

For these samples, I suspended 3.5 g soil in 18 ml demineralized water. This soil-to-water ratio is generally adopted for soil analyses, because it allows enough liquid for even extremely absorbent soils. The salinity, the conductivity and the pH of the bottom suspensions were measured. Semiquantitative determinations of total hardness and the concentrations of calcium, potassium and sulfate were made with Merckoquant test sticks.

The abiotic factors are compiled for the different samples in the Table 1. The salt contents range from 12‰ to 32‰, and the pH values lie between 8.5 and 9.5. These results are in quite good agreement with the values given by Williams and Buckney (1976) for the waters in the Coorong.

After the measurements had been made, each soil suspension was divided among three Petri dishes and kept at room temperature. After one to three days, the first species had excysted. The ciliates were taken directly from these raw cultures for examination. The same procedure was followed for samples from the field. To clarify special questions, pure cultures of individual species were also prepared.

3.2. Taxonomic investigation

The ciliates were first observed closely in the living state. The infraciliature was revealed by the protargol silver impregnation method of Wilbert (1975) and by dry silver impregnation according to the method of Klein (1927). The systematic assignments of the ciliates were made in conformity with Corliss (1979). Within the class Polyhymenophora Jankowski, 1967, I followed Hemberger (1982) in classifying the Hypotrichida.

3.3. Deposition of type material

The holotype preparations of the new species described here are deposited in the Zoological Institute of the University of Bonn, Poppelsdorfer Schloß.

4. RESULTS

Of the 96 ciliate species found, 11 species will be described in the following section. Some of these are ciliates new to science; in the case of the others, it seemed desirable to extend their descriptions, or there are discrepancies and inconsistencies in the data published previously.

4.1. Description of selected species

The silvering techniques mentioned above allow species identification. Equally important to the taxonomist is that, apart from characters observable only in life, most morphological characters can be accurately measured in animals prepared in this way, so that statistical evaluation is possible.

Spathidium macrostomum sp. n.(Kinetofragminophora: Gymnostomata) (Fig. 5, Table 6)

Diagnosis: in vivo 110-150 µm long *Spathidium* with oral ridge bent into a hatchet shape, more than 100 macronuclear segments and a simple terminal vacuole; soma kineties 21-26 on left, 14-17 on right. Circumoral kinety not separated from the soma kineties left-laterally, separated only in sections right-laterally.

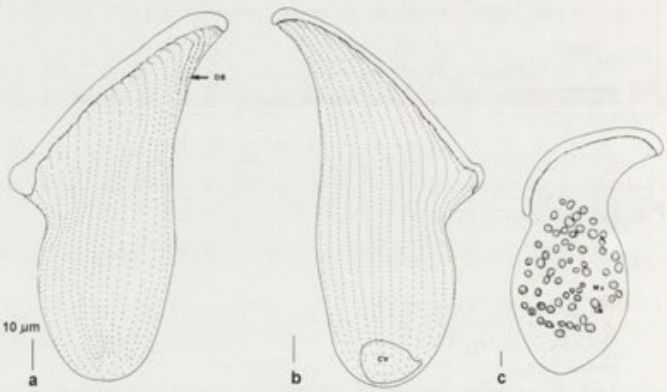

Fig. 5. *Spathidium macrostomum* sp. n. after protargol impregnation (a-c). a, b - infraciliature of the left and right side. c - macronucleus. Abbreviations: CV - contractile vacuole, DB - dorsal brush, Ma - macronucleus

Table 1

Abiotic factors at individual sample sites

Sample	Salinity ‰	Cond.^{mS}/cm	pH	SO_4^{2-}, mg/l	K^+, mg/l	Ca^{2+}, mg/l	alk. earths, °d*
1	25.0	38.5	9.5	900-1400	700	100	23
2	25.5	40.5	8.9	900-140	700	250	23
3	32.0	50.0	8.5	900-1400	700	200	23
4	12.0	20.0	8.5	900-1400	700	200	23

*Sum of all Ca and Mg ions; 1° = 10 mg/l CaO

Locus typicus: numerous in autumn between algal mats in the lakes Manito and Little Manitou (Saskatchewan, Canada).

Morphology: overall broadly jug-shaped, trunk slightly flattened, neck thin and translucent. Sturdy, well demarcated oral ridge, the edges of which are densely packed with extrusomes *ca* 4 µm long. It is set at an angle of *ca* 50° to the long axis of the trunk. 100 or more spherical macronuclear segments, among which the micronuclei cannot be discerned. Soma cilia *ca* 10 µm long, those of the dorsal brush (DB) *ca* 4 µm. Locomotion: slow, weakly contractile. Food: other ciliates. Infraciliature typical of the genus (Foissner 1984).

Species comparison: the family Spathidiidae has very recently received thorough taxonomic investigation Dragesco and Dragesco-Kerneis (1979), Foissner (1981, 1984), Foissner and Didier (1981). *S. macrostomum* sp. n. has the largest oral ridge of all known *Spathidium* species. Such a long, wide oral ridge is otherwise found only in species of the genus *Epispathidium* Foissner, 1984. Hence it is remarkable that a *"Spathidium"* with this character is not classified as *Epispathidium*. However, there is a clear distinguishing character: species of *Epispathidium* have an isolated circumoral kinety (cK), distinctly separated from the soma kineties.

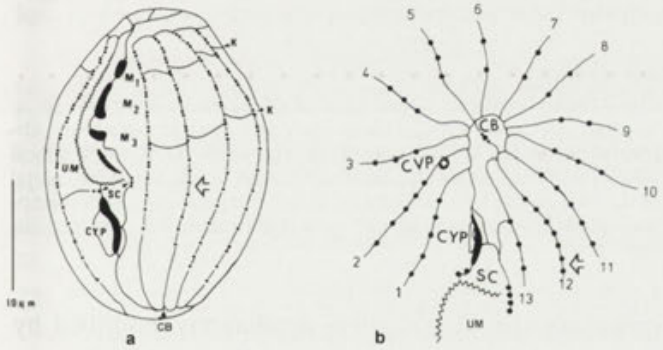

Fig. 6. *Cyclidium glaucoma* by wet silver impregnation. a - ventral infraciliature, b - caudal pole. The arrow indicates the condensation of basal bodies in kinety N-1. Abbreviations: CB - polar basal body complex, CVP - pore of the contractile vacuole, CYP - cytoproct, K - commissural silverline, M_{1-3} - membranelles, SC - scuticus, UM - undulating membrane, 1-13 - kineties

Cyclidium glaucoma Müller, 1786 (Oligohymenophora: Scuticociliatida) (Fig. 6, Table 7)

Diagnosis: in vivo 21-30 x 18-22 µm. Broadly ovoid with wide frontal plate. Thirteen kineties and a caudal cilia half the length of the body. Macronucleus (Ma) spherical with adjacent micronucleus (Mi). Contractile vacuole (CV) in the posterior quarter with an excretory pore (CVP) at the end of kinety 2. Locomotion saltatory with short rest pauses during which the cilia are spread out and bacteria swept into the mouth. Undulating membrane (UM) half the length of the body. The species is common in the lakes Manito, Deadmoose and Muskiki (Saskatchewan) at S from 29.9 to 370.2‰.

Comparison with populations of Champaign-Urbana, Death Valley and Bonn shows that the Saskatchewan population has the largest individuals, with the greatest number of kineties. The cyclidiids in the salt lakes have another character typical of the European *C. glaucoma* populations: that the kinetosomes become denser in the posterior half of the N-1 kinety (Klein 1927, Parducz 1940, Didier and Wilbert 1981, Bardele 1983). Also typical of the species is the last (N) kinety, which runs caudally to the cytoproct (CYP), and the directly connecting silverline system of the N and N-1 kineties, that separately join the caudal circular fibril.

The comparison shows that salt lakes are inhabited by a special type of *C. glaucoma*.

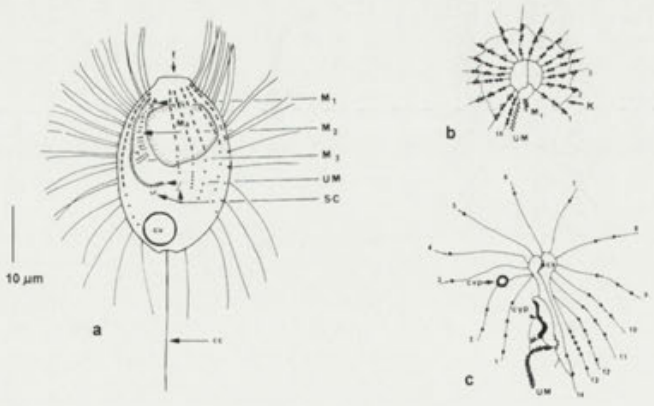

Fig. 7. *Cyclidium citrullus*. a - ventral view from life and after protargol impregnation. b - anterior, c - caudal pole after wet silver impregnation. Abbreviations: CC - caudal cilium, CV - contractile vacuole, CVP - contractile vacuole pore, CYP - cytoproct, f - frontal plate, K - commissural silverline, M_{1-3} - membranelles, Ma - macronucleus, SC - scuticus, UM - undulating membrane

Cyclidium citrullus Cohn, 1866 (Fig. 7, Table 8)

Diagnosis: *in vivo* 21-27 µm long x 14-18 µm wide, ovoid. Usually 15 kineties. Undulating membrane about 2/3 body length. The caudal cilium is seated in a distinct indentation. Easily recognizable in life by a depression that extends the oral cavity to the posterior end and into which the undulating membrane is folded. Locomotion includes longer rest pauses than that of *C. glaucoma*.

The species is also widespread in our latitudes, and its ecology has been well studied (Bick 1966, Bick and Kunze 1971). The silverline system was examined by Czapik (1963); her illustration matches my findings (Figs. 7b, c), but gives no evidence as to the infraciliature. Details of the latter are made visible by protargol silver staining. Typical of the species is the short last left kinety (N) terminating next to the UM. The next kineties, N-1, -2, and -3, are negligibly longer. Here again (as in *C. glaucoma*) there is a notable density of kinetosomes in the N-1 kinety.

The directly connecting silverline of the N- kinety does not open into the caudal circular fibril but instead is in direct communication with the basal-body complex (CB) of the caudal cilium (CC).

The *C. citrullus* collected by Kahan (1972) from hot springs at Tiberias (Israel) differs in all respects from the populations studied by Czapik and me. With its 10 kineties, the simple form of the N-1 kinety, and the proportions of the membranelles (M1-3), it most closely resembles the *C. glaucoma* populations described by Berger and Thompson (1960) and Blacker (1975).

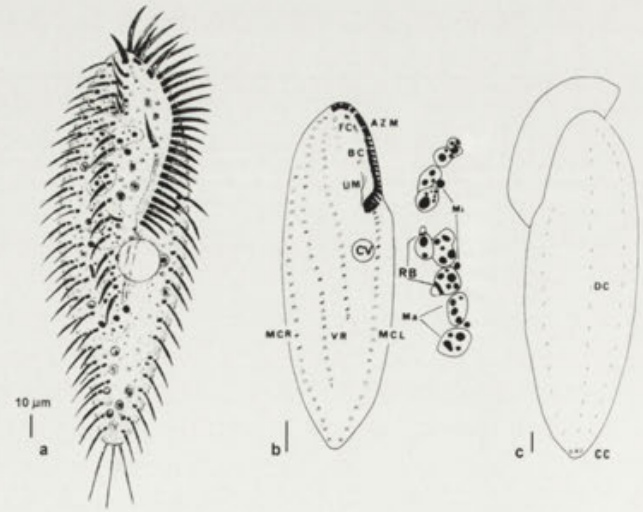

Fig. 8. *Cladotricha halophila* sp. n., a - ventral view from life; b, c - nuclear apparatus, ventral and dorsal infraciliature from protargol-impregnated specimens. Abbreviations: AZM - adoral zone of membranelles, BC - buccal cirrus, CC - caudal cirri, CV - contractile vacuole, DC - dorsal kineties, FC - frontal cirri, Ma - macronucleus, MCR, MCL - right and left marginal cirri, Mi - micronucleus, RB - reorganization band, UM - undulating membranes, VR - ventral row of cirr

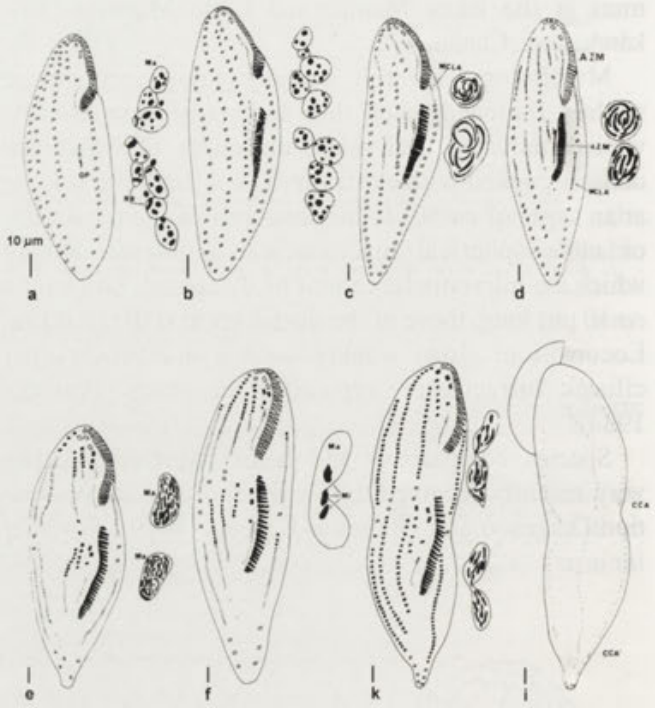

Fig. 9. Morphogenetic stages (a-h) of *Cladotricha halophila* sp. n. Ventral and dorsal infraciliature from protargol preparations. Abbreviations: AZM - adoral zone of membranelles, AZM' - new adoral zone of membranelles in the opisthe, CCA, A' - anlagen of the caudal cirri, MCLA, MCLA' - anlagen of the left marginal cirri, Ma - macronucleus, Mi - micronucleus, OP - oral primordium, RB - reorganization band

Cladotricha halophila sp. n. (Polyhymenophora: Hypotrichida) (Figs. 8, 9; Table 9)

Diagnosis: *in vivo* 102-172 x 25-39 µm, spindle-shaped *Cladotricha* with *ca* 10 Ma, 3 CC, 2 ventral rows of cirri (VR) and 3 dorsal kineties (DC). Frontal cirri (FC) conspicuously thickened.

Locus typicus: common in the soil on the dry bottom of a salt lake in Coorong, Australia.

Morphology: front end slightly, hind end strongly tapered and pointed. The first 3 FC are thicker than the other two-kinety cirri. The membranes composed of paired kinetosomes, the paroral and adoral membranes, differ in length. Dorsal ciliature comprises 3 kineties, ending in 3 CC. The dorsal cilia are 4 µm long, the CC 12 µm.

Ma are arranged along the median axis of the animal. Usually there are 3 Mi. CV in the standard position, dorsal ahead of the middle. In culture the swimming

ciliate rotates slowly about its long axis. Jerking suddenly forward and backward, it explores the substrate for food (bacteria). The body is particularly flexible and changeable in shape in the frontal region. The cytoplasm is light and colourless, with fine inclusions. The posterior end often appears dark; evidently the excretory vacuoles assemble here.

In the cultures the species was present at S of 24-66‰ and a pH between 8.1 and 9.5.

At the onset of morphogenesis (Fig. 9), a narrow oral primordium (OP) appears on the ventral surface, between the left marginal row (MCL) and the second VR; in the Ma segments reorganization bands (RB) form.

Beginning at OP (Fig. 9a, b) basal bodies proliferate frontally and caudally, forming an elongated field of kinetosomes. The first membranelles of the new adoral membranelle zone (AZM') are already forming. To the right of them 2 anlagen have developed. The buccal cirrus (BC) and UM of the proter have also reoriented. The macronuclear segments have enlarged; Mi are no longer detectable.

The frontal part of the adoral membranelle zone (AZM) of the opisthe has almost fully differentiated (Fig. 9c). To the right of it a third anlage has formed. Cirri of 2 VR have differentiated into anlagen at two places, as have a few FC of the proter. In parallel with these, anlagen develop from cirri in MCL. The macronuclei have fused to 2 nuclear complexes, within which are streaks of chromatin.

The new AZM is now completely formed (Figs. 9d, e). Anlagen have appeared at four places in the marginal rows (MCRA,A', MCLA,A'). In both the anterior and the posterior fission product the first FC have formed. The genesis of the first FC could not be followed in detail. It presumably arises from anlagen I, along with UM. The cirri of the 2 VR are also beginning to differentiate, proceeding caudally from the front.

Cirri are formed in the same way in the marginal rows (Figs. 9f, g). FC and the adoral and paroral membranes of both fission products have developed. Finally, all the new cirri are present; except for AZM of the proter, all "old" cirri have been replaced. A dividing Ma is present in both proter and opisthe. Mi are not discernible.

Two anlagen form in each of 3 DC (Fig. 9h). At their ends the anlagen of the caudal cirri (CCA,A') of the two fission products are visible.

I have assigned this species to the genus *Cladotricha* Gajewskaja, 1925 as a new species. Distinguishing characters of this genus: presence of a right (MCR) and a left marginal row (MCL), 1 to 3 VR, and FC arranged

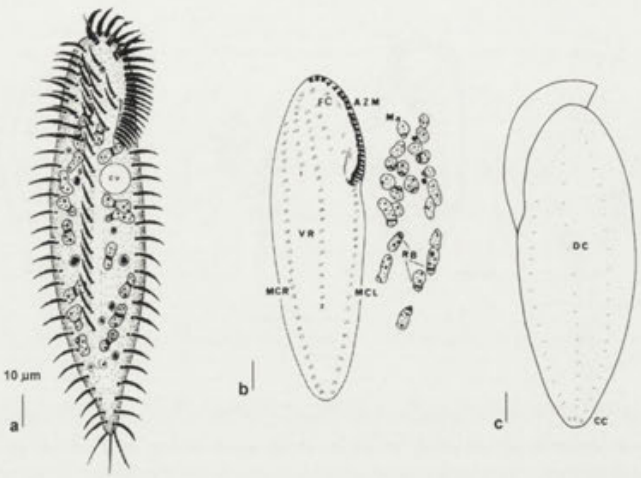

Fig. 10. *Cladotricha edaphoni* sp. n. from life, ventral (a) and after protargol impregnation (b, c). Ventral infraciliature and nuclear appa-ratus (b), infraciliature in dorsal view (c). Abbreviations: AZM - adoral zone of membranelles, CC - caudal cirri, CV - contractile vacuole, DC - dorsal kineties, FC - frontal cirri, Ma - macronucleus; MCR, MCL - right and left marginal cirri, RB - reorganization band, VR - ventral cirri.

in two groups. Transverse cirri (TC) are lacking. TC arise from 2 longitudinal anlagen.

VR develop from longitudinal anlagen within the existing rows. A feature inconsistent with the genus diagnosis of Hemberger (1982) is the absence of isolated adoral membranelles in the frontal region of the membranelle zone.

Species comparison: Species previously assigned to this genus is *Cladotricha koltzowii*, described by Gajewskaja (1925), who discovered it in a salt lake near Sevastopol. This *Cladotricha* is more worm-like in shape and has a simple macronucleus.

Cladotricha edaphoni sp. n. (Fig. 10, Table 10)

Diagnosis: *in vivo* 90-125 x 32-49 µm *Cladotricha*, shaped like a slender spindle with 15-20 Ma segments, 2 VR, 3 DC and 3 CC.

Locus typicus: in the soil of the dry bottom of a salt lake in the Coorong, Australia.

Morphology: elongated shape, length:width 3:1, pointed frontally and caudally. The right one of 2 ventral rows (VR1) is short, ending at the level of the peristome, while the left one (VR2) extends into the posterior third of the body. Adoral membrane conspicuously shorter than paroral. The cirri of the marginal rows are *ca* 10 µm long. FC thickened. Dorsally 3 rows of kineties, terminat-

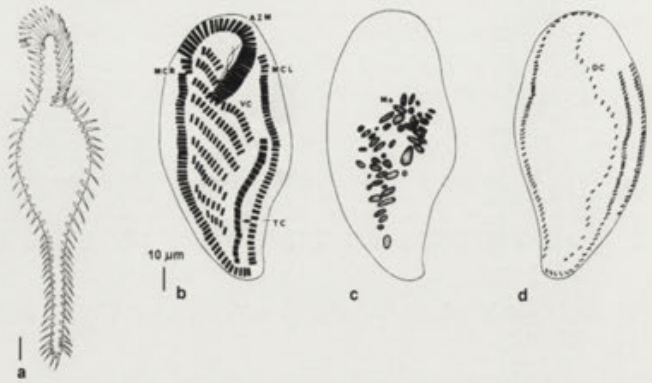

Fig. 11. *Epiclintes ambiguus*. a - from life, b - infraciliature ventral and c - dorsal in cells contracted by fixation. Abbreviations: AZM - adoral zone of membranelles, DC - dorsal kineties, Ma - macronucleus; MCR, MCL - right and left marginal cirri, TC - transverse cirri, VC - ventral cirri. Arrow indicates

ing in 3 CC. The latter are about 15 mm long and project well beyond the hind end. In the middle of the body are 4 Mi along with many Ma. CV postoral in the standard position. *C. edaphoni* has a very changeable shape. The cytoplasm is light and translucent. Numerous individuals develop at relatively high salt concentrations of S = 26-57‰. The pH of the soil suspension was 8.8.

Epiclintes ambiguus Müller, 1786 (Fig. 11, Table 11)

Genus diagnosis: ventrally 1 MCL and 1 MCR, as well as many diagonal rows of cirri. No differentiated FC. TC present. No CC. Body elongated, subdivided into "head" and "tail", extremely contractile.

I agree with Borror (1972) and Hemberger (1982), who regard all species so far described as equivalent to *E. ambiguus* Müller.

Syn.: *Trichoda ambiguus* Müller, 1786; *Oxytricha ambigua* Bory de St. Vincent, 1826; *Oxytricha auricularis* Claparède & Lachmann, 1868; *Epiclintes auricularis* Stein, 1859; *Epiclintes vermis* Gruber, 1888; *Epiclintes pluvialis* Smith, 1900.

Discussion: the population found in Lake Qarun corresponds substantially to the descriptions by Kahl (1930-35) and Wicklow and Borror (1990). Peristome part and tail-like hind end well demarcated from trunk. Extended as long as 200 µm, contracted *ca* 80 µm. The cirri of MCR and MCL and those of VC are identical in structure and are seated on 2 kineties, each *ca* 8 kinetosomes long. TC are distinctly thicker and are based on 3 kineties. In life the species is noticeable by its very sudden appearance; at the slightest disturbance it responds by contracting and adhering to the substrate like a suction cup so that it can no longer be observed.

The list of synonyms suggests that different populations are extremely variable. For this reason I made detailed measurements of the specimens from Lake Qarun, in order to compare them with those from the Atlantic (Wicklow and Borror 1990). Such a comparison was not possible for the other populations. The Table 11 shows that the main differences are in size. It is unfortunate that Wicklow and Borror (1990) did not investigate their population biometrically; it must therefore remain in doubt whether these are indeed populations of a single species.

Euplotes pterotae sp. n. (Fig. 12, Table 12)

Diagnosis: *in vivo* 95-193 x 78-138 µm. 10 frontoventral cirri, 5 TC and 5-7 CC. Ten DC. Posterior left edge of body expanded like a wing. Dorsal silverline system of the "single-vannus type" (Curds 1975).

Locus typicus: common between the algal mats of the littoral in Lake Qarun.

Morphology: this species, extremely variable in size and shape, is characterized in particular by the flat, winglike expansion of the left edge of the body and by the arrangement of CC in two groups. Body form a broad to very broad oval, slightly convex dorsally, with 7 indistinct ribs. Shape of Ma not constant, usually as illustrated or C-shaped. A total of 10 DC. In only

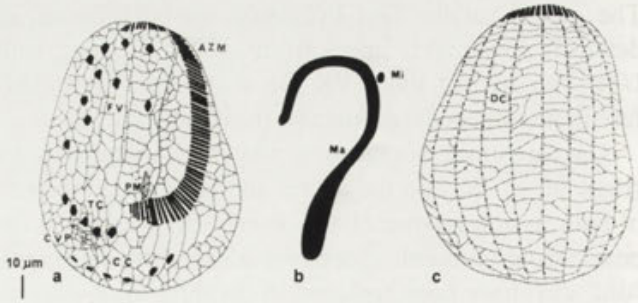

Fig. 12. *Euplotes pterotae* sp. n., a - ventral silverline system, b - nuclear apparatus, c - dorsal silverline system after wet silver impregnation. Abbreviations: AZM - adoral zone of membranelles, CC - caudal cirri, CVP - contractile vacuole pore, DC - dorsal kineties, FV - frontoventral cirri, Ma - macronucleus, Mi - micronucleus, PM - paroral membrane, TC - transverse cirri

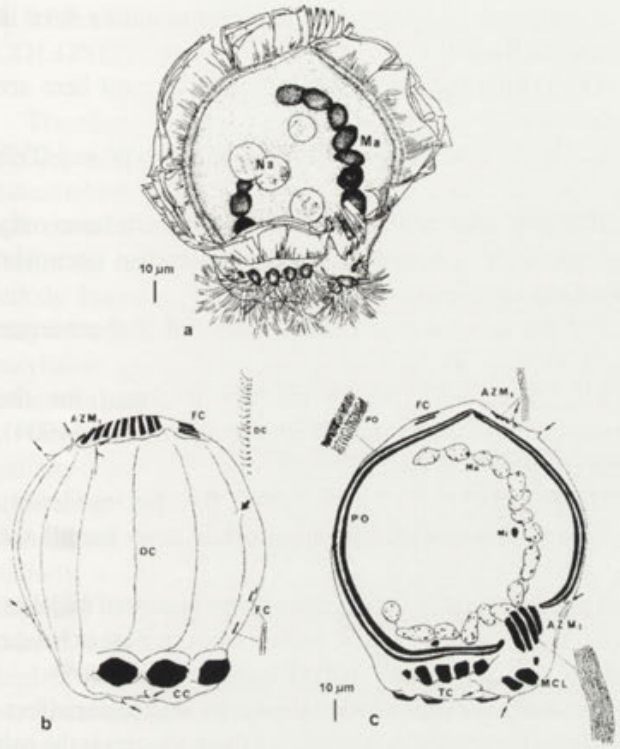

Fig. 13. *Uronychia magna.* a - ventral view from life, b - dorsal und c - ventral in infraciliature after protargol impregnation. AZM_{1-2} - adoral zone of membranelles, CC - caudal cirri, DC - dorsal kinetis, FC - frontal cirri, Ma - macronucleus, MCL - marginal cirri Mi - micronucleus, Na - food vacuole, PO - polykinety, TC - transverse cirri. The arrows indicate the thorns a frequently observed interruption in the 1st right dorsal kinety

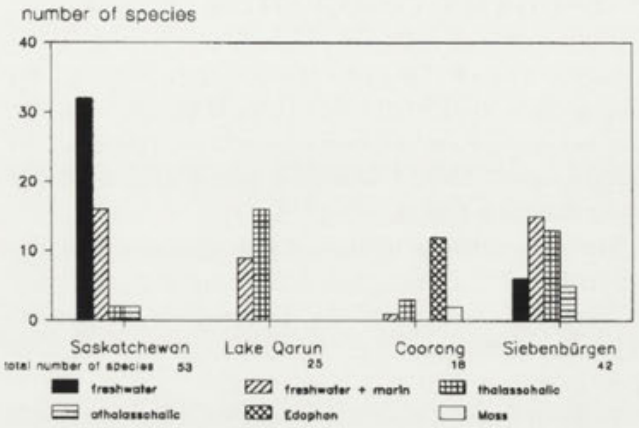

Fig. 14. Structure and comparison of the ciliate coenoses in Saskatchewan (Canada), Lake Qarun (Egypt), Coorong (Australia) and Siebenbürgen Entz (1904)

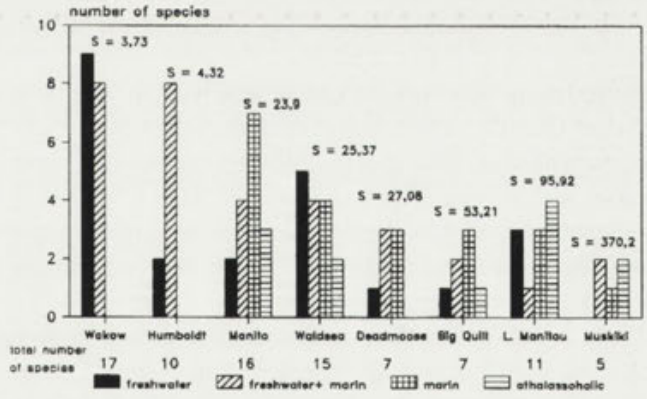

Fig. 15. The structure of the ciliate coenoses of the lakes in Saskatchewan in order of increasing salinity. S - salinity

slightly expanded individuals 1 DC can be situated ventrally, on the left lateral surface. In DC there are *ca* 20 cilia. Peristome about three-quarters of length of body, slightly concave. Paroral membrane (PM) rhomboid, composed of several short kineties. In AZM there are 56-72 membranelles. The dorsal silverline system is of the type of *E. vannus*: see Tuffrau (1960) and Curds (1975). The great variability of shape in this species may be a means of defense against a predator. A morphological change can be induced in *Euplotes octocarinatus* by its predator *Lembadion lucens*, as described by Kuhlmann and Heckmann (1985). This phenomenon could be a cause of the pronounced form variance in *Euplotes* species in general.

Species comparison: *E. pterotae* in outline resembles *E. novemcarinata* Wang, 1930. The latter species lives in freshwater and is 60-75 µm long, but its infrastructure is completely different. Nothing is known about its silverline system. Among the *Euplotes* species with *"vannus"* silverline system there are certain resemblances to *E. mutabilis* Tuffrau, 1960, in that the strict rectangular pattern of *"vannus"* is disrupted by loops and connections between the kineties.

Uronychia magna Pierantoni, 1909 (Fig. 13, Table 13)

This species, described from the Gulf of Naples, has the largest body size in the genus: up to 450 µm according to Pierantoni (1909) and over 300 µm in my own observations. It had not been encountered again since it was first described. I found it regularly among algae in the littoral of Lake Qarun. Biometry and infraciliature follow.

5. STRUCTURE AND COMPARISON OF THE INVESTIGATED COMMUNITIES

Now we turn to the population ecology of the ciliate communities considered here. It should be noted that the species composition of these communities represents the autumn aspect for the Canadian lakes and the spring aspect for Lake Qarun.

The data relating to the ciliate populations of the individual habitats to be compared here are assembled in Tables 2-5 and Figs. 14, 15. To complete the picture, additional findings of Entz (1904) from salt lakes in Siebenbürgen will also be considered.

To document that the coenoses analyzed here are intact, their species/genera ratio will be used as a criterion. It would be expected to be close to 1, because species of a given genus have very similar food requirements and are thus in direct competition; it is a general rule that in established animal communities, each genus is represented only by the single species that has proved superior to the others under the conditions prevailing in the biotope (Schwerdtfeger 1975).

To answer the question of the origin of the ciliate fauna, in Fig. 14 the species of the communities are grouped according to their characteristic habitats. It is clear that the Canadian salt lakes contain primarily freshwater or euryhaline ciliate species, whereas in Lake Qarun marine species dominate. The saline bodies of water in Siebenbürgen, relict of Tertiary residual seas, retain a salt composition like that of the ocean; here the predominant species are marine and euryhaline.

The ciliate community in the soil samples from dry lakes in the Coorong has a special position. It is an edaphic community. 15 out of the total of 18 species identified are typically soil-dwellers or, like *Spathidium muscicola* and *Homalogastra setosa*, inhabit the capillary water of mosses.

As a measure with which to compare the species compositions of communities with one another, Jaccard 1902 has defined the "species identity" I_s (also known as the community coefficient or Jaccard's Number) according to the following equation:

$$I_s = \frac{g}{a + b - g} \times 100$$

where: a - number of species in community 1,
b - number of species in community 2,
g - number of species the two communities have in common (Bick 1989).

The results for the communities of interest here are as follows:

I_s for the coenoses of Canada and Egypt = 4.05% (g= 3).

The coenoses of Canada and Egypt each have only one species in common with the Australian coenosis: *Uronema nigricans*.

For the coenoses in Lake Qarun and Siebenbürgen I_s = 4.8% (g = 3).

The "greatest" species identity is found for the coenoses of Canada and Siebenbürgen Entz (1904), where I_s = 10.4% (g = 9).

Hence, apart from the last value, the species identity or community coefficient approaches zero for all the coenoses considered here.

This result reflects the origin and prior history of the lakes, which led to athalassohaline sulfate lakes in Saskatchewan and a thalassohaline lake in the Fayum Depression.

According to Bick (1961, 1964a, b), one factor affecting the salinity-tolerance of many ciliate species is the salt composition of the water; that is, various species have a lower "limiting concentration" in brackish water of the sodium type than in marine brackish water. The salinity-tolerance of individual species is of crucial significance for the colonization of saline lakes, determining the structure of the individual coenoses. The resulting difference is also apparent in a comparison of total species numbers - the athalassohaline lakes are characterized by a species deficit (Fig. 15).

There is an even greater deficit in the edaphic ciliate community inhabiting the dry bottoms of the Coorong lakes. Here 18 species were found, compared with 40 species in a savanna soil (Buitkamp 1977) and ca 70 in the edaphon of the Großglockner region of Austria (Foissner 1978). Salt is evidently a significant ecological factor in the colonization of soil as well as lakes. Furthermore, species density is likely to depend on the relative amounts of the different salts as well as on seasonal differences in salt content and water temperature. These aspects demand further study.

The data from Saskatchewan shown in Fig. 14 are analyzed separately for the individual lakes in Fig. 15. Clearly, the colonization of the lakes reflects the Second Biocoenotic Principle of Thienemann (1913), according to which biased and extreme living conditions drastically reduce the number of species. The upper limit of salinity for many freshwater and euryhaline (tolerant of both fresh and sea water) species is 25‰.

6. RELATIONSHIPS BETWEEN SALINITY AND COLONIZATION BY CILIATES

The ciliate communities in the Canadian lakes exemplify the Second Biocoenotic Principle of Thienemann (1913) for extreme biotopes: there are fewer species in lakes with higher salt concentration. But what are the relative numbers of freshwater and euryhaline species, in comparison with the strictly thalassohaline or athalassohaline species? From this ratio one can see how the decrease in number of freshwater and euryhaline species with rising salinity is accompanied by an increase in pure saltwater species (Table 14).

Disregarding the value at S = 23.9‰ (Manito Lake), as salinity rises the ratio becomes progressively smaller, indicating relatively more saltwater species. Their dominance in Manito Lake, despite its relatively low salinity, is due partly to the special ionic composition of this lake (Fig. 3) but mainly reflects the varying salinity-tolerance of the individual species. At S of 27.08‰ or higher, there are fewer species in all the groups considered.

According to Remane (1971), when the numbers of species in bodies of water are plotted as a function of increasing salinity of the water, a complicated curve results. From the value for fresh water it first falls to a minimum at 5-100‰ then rises to a maximum at 300‰ from which it falls again. The ciliate communities in the salt lakes of Saskatchewan conform to the same pattern.

From the graph in Fig. 14 it can be seen that in the coenoses of Saskatchewan and Siebenbürgen, euryhaline species predominate. Even in Lake Qarun, nearly half the species encountered are in this category.

By comparing the lists of species inhabiting fresh water, salt water and both habitats, it is possible to identify typical species for each of the coenoses, called "differential species" by Schwerdtfeger (1975). The differential species of the athalassohaline lakes are found to be *Bakuella marina, Bakuella imbricata, Diophrys appendiculata, Fabrea salina* and *Uronychia transfuga*. Differential species in the thalassohaline Lake Qarun are *Condylostoma reichi, Strombidium kahli, Trachelostyla spiralis* and *Uronema filificum*. So far the only data available for ciliate coenoses in saline soils have been provided by Pomp and Wilbert (1988). Differential species for this community are *Pseudocohnilembus persalinus* and *Uronema nigricans*.

Cyclidium glaucoma and *Cyclidium citrullus* are useful indicators of water quality. In the case of the first of these, it was important to determine whether the type of *C. glaucoma* living in the salt lakes is a special morphological variant. Biometric comparison, as in Table 6 confirms that. Knowing how the saline form compares with the other *C. glaucoma* variants, one can make better use of it as an indicator. These are the first biometric data published for

APPENDIX

Table 2

Systematic list of identified ciliates (system of Corliss 1979). Occurrence (+)

	Egypt	Australia	Canada
Phylum **CILIOPHORA**			
Class **Kinetofragminophora**			
Subclass **Gymnostomata**			
Order **Karyorelictida**			
Remanella rugosa	+	.	.
Remanella faurei	+	.	.
Order **Prostomatida**			
Holophrya simplex	.	+	.
Prorodon rabei	.	.	+
Order **Haptorida**			
Actinobolina radians	.	.	+
Lacrymaria olor	.	.	+
Mesodinium pulex	.	.	+
Spathidium macrostomum sp. n.	.	.	+
Spathidium muscicola	.	+	.
Trachelius ovum	.	.	+
Order **Pleurostomatida**			
Hemiophrys pleurosigma	.	.	+
Litonotus lamella	.	.	+
Loxophyllum setigerum	+	.	.
Subclass **Hypostomata**			
Order **Synhymenida**			
Chilodonella uncinata	.	.	+
Chilodontopsis depressa	.	.	+
Orthodonella hamatus			
Trithigmostoma cucullus	.	.	+
Order **Nassulida**			
Nassula notata	.	.	+
Drepanomonas revoluta	.	.	+
Subclass **Vestibulifera**			
Order **Colpodida**			
Cyrtolophosis mucicola	.	.	+
Sagittaria australis	.	.	+
Class **Oligohymenophora**			
Subclass **Hymenostomata**			
Order **Hymenostomata**			
Espejoia culex	.	.	+
Espejoia mucicola	.	.	+
Frontonia leucas	+	.	.

Table 2 (cont.)

Order **Scuticociliatida**			
Cinetochilum australis	.	+	.
Cinetochilum margaritaceum	.	.	+
Cyclidium citrullus	+	.	.
Cyclidium glaucoma	.	.	+
Cyclidium sp.	.	+	.
Homalogastra setosa	.	+	.
Loxocephalus luridus	.	.	+
Paranophrys thompsoni	.	.	+
Parauronema virginianum	.	.	+
Pleuronema coronatum	+	.	+
Pleuronema marinum	+	.	.
Pseudocohnilembus marinus	.	.	+
Pseudocohnilembus persalinus	.	+	.
Subclass **Peritricha**			
Order **Peritrichida**			
Ophrydium versatile	.	.	+
Vorticella microstoma	.	.	+
Class **Polyhymenophora**			
Subclass **Spirotricha**			
Order **Heterotrichida**			
Blepharisma sp.	.	+	.
Condylostoma patulum	+	.	.
Condylostoma reichi	+	.	.
Condylostoma vorticella	.	.	+
Fabrea salina	.	.	+
Stentor polymorhus	.	.	+
Order **Oligotrichida**			
Halteria grandinella	.	.	+
Strobilidium humile	.	.	+
Strombidium styliferum	.	.	+
Strombidium kahli	+	.	.
Order **Hypotrichida**			
Aspidisca aculeata	+	.	.
Aspidisca cicada	.	.	+
Aspidisca dentata	.	.	+
Aspidisca lynceus	.	.	+
Aspidisca pulcherrima	+	.	.
Bakuella edaphoni	.	+.	
Bakuella imbricata	.	.	+
Bakuella marina	.	.	+
Cladotricha edaphoni sp. n.	.	+	.
Cladotricha halophila sp. n.	.	+	.
Diophrys appendiculata	.	.	+
Diophrys scutum	+	.	.
Epiclintes ambiguus	+	.	.
Euplotes balteatus	.	.	+
Euplotes charon	+	.	.
Euplotes elegans	.	.	+
Euplotes inkystans	.	.	+
Euplotes novemcarinata	+	.	.
Euplotes woodruffi	+	.	.
Euplotes pterotae sp. n.	+	.	.
Euplotes sp.	.	.	+
Gonostomum affine	.	+	.
Holosticha diademata	+	.	+
Holosticha gelei	.	.	+
Paraurostyla weissei	.	.	+
Stylonychia mytilus	.	.	+
Tachysoma pellionella	.	.	+
Trachelostyla spiralis	+	.	.
Uronychia magna	+	.	.
Uronychia transfuga	+	.	.

Table 3

Occurrence (+) and origin of the ciliates collected in Saskatchewan (system of Corliss 1979) compared on occurrence of species compiled from Kahl (1930-35), Bick and Kunze (1971) and Wilbert and Kahan (1981)

Lake:	1	2	3	4	5	6	7	8	f	ath	th
Class: **Kinetofragminophora**											
Subclass: **Gymnostomata**											
Actinobolina radians **P**	.	.	.	+	.	.	.	.	+	.	.
Amphileptus pleurosigma	.	.	.	+	.	.	.	.	+	.	+
Lacrymaria olor	.	.	.	+	.	.	.	.	+	.	+
Litonotus lamella	+	+	+	+	.	.	.	+	+	+	+
Mesodinium pulex	.	.	.	+	.	.	.	.	+	.	+
Prorodon raabei	.	.	+	.	.	.	.	.	.	.	+
Spathidium macrostomum sp. n.	.	.	+	.	.	.	+	.	.	+	.
Trachelius ovum	+	.	.	.	.	.	.	.	+	.	.
Subclass: **Vestibulifera**											
Cyrtolophosis mucicola	.	+	.	.	.	.	.	.	+	+	.
Subclass: **Hypostomata**											
Chilodonella uncinata	.	+	.	.	.	.	.	.	+	+	+
Chilodontopsis depressa	.	.	.	.	.	.	.	+	.	+	
Drepanomonas revoluta	.	+	.	.	.	.	+	.	+	.	.
Orthodonella hamatus	.	.	.	.	+	.	.	.	.	.	+
Nassula notata	.	.	+	+	.	.	.	.	.	.	+
Trithigmostoma cucullulus	+	+	.	.	.	.	.	.	+	+	+
Class: **Oligohymenophora**											
Subclass: **Hymenostomata**											
Cinetoch. margaritaceum	+	+	.	.	.	.	.	.	+	+	+
Cyclidium glaucoma	.	.	+	.	.	+	.	+	+	.	+
Espejoia culex	+	.	.	.	.	.	.	.	+	.	.
Espejoia mucicola	+	.	.	.	.	.	.	.	+	.	.
Loxocephalus luridus	.	.	.	+	.	.	.	.	+	.	.
Paranophrys thompsoni	+	.	.	.	.	.	.	.	+	.	.
Parauronema virginianum	.	.	+	.	.	.	.	.	+	+	+
Pleuronema coronatum	.	.	+	.	+	+	+	.	+	.	+
Pseudocohnilembus marinus	.	.	.	.	.	.	.	+	.	.	+
Tetrahymena rostrata	+	.	.	.	.	.	.	.	+	.	.
Uronema marinum	.	.	.	.	.	+	.	.	+	+	+
Uronema nigricans	+	.	.	.	+	.	.	.	+	+	+
Subclass: **Peritricha**											

Table 3 (cont.)

Ophrydium versatile	+	.	.	.	.	.	.	.	+	.	.
Vorticella microstoma	.	.	.	+	.	.	.	.	+	.	.
Class: **Polyhymenophora**												
Subclass: **Spirotricha**												
Order: **Heterotrichida**												
Condylostoma vorticella **P**	.	.	.	+	.	.	.	.	+	.	.
Fabrea salina **P**	.	.	.	.	.	.	+	.	.	+	+
Stentor polymorphus	+	.	.	.	.	.	.	.	+	.	+
Order: **Oligotrichida**												
Halteria grandinella **P**	+	+	.	.	.	.	.	.	+	.	+
Strobilidium humile **P**	+	.	.	.	.	.	.	.	+	.	.
Strombidium styliferum **P**	.	.	.	.	.	.	+	.	.	.	+
Order: **Hypotrichida**												
Aspidisca cicada	.	+	.	.	.	.	.	.	+	+	+
Aspidisca dentata	.	.	+	+	.	.	.	.	.	.	+
Aspidisca lynceus	.	+	.	.	.	.	.	.	+	.	.
Bakuella marina	.	.	+	+	.	+	+	.	.	+	.
Bakuella imbricata	.	.	+	.	.	.	+	.	+	.	.
Diophrys appendiculata	.	.	.	.	+	+	+	.	.	.	+
Euplotes sp.	.	.	.	.	.	.	.	+	.	.	.
Euplotes balteatus	.	.	+	.	.	.	+	.	.	.	+
Euplotes elegans	.	.	+	+	.	.	.	.	.	.	+
Euplotes inkystans	.	.	+	+	+	+	.	.	+	.	
Euplotes elegans	.	.	+	+	.	.	.	.	.	.	+
Euplotes inkystans	.	.	+	+	+	+	.	.	+	.	.
Gonostomum affine	.	.	.	.	.	.	+	.	+	.	.
Holosticha diademata	.	.	+	.	.	.	.	+	+	+	+
Holosticha geleii	.	.	+	.	.	.	+	.	.	+	.
Parurostyla weissei	+	.	.	.	.	.	.	.	+	.	.
Stylonychia mytilus	+	.	.	.	.	.	.	.	+	.	+
Tachysoma pellionella	+	+	.	.	.	.	.	.	+	.	+
Uronychia transfuga	.	.	+	+	+	+	+	.	.	.	+
Urostyla sp.	.	.	.	+	.	.	.	+	.	+	.

Lake numbers: 1 - Wakow, 2 - Humboldt, 3 - Manito, 4 - Waldsea, 5 - Deadmoose, 6 - Big Quill, 7 - Little Manitou, 8 - Muskiki.
Abbreviations: f - freshwater, ath - athalassic saline, th - thalassic saline. Habitat: P - plankton, all other species belong to the periphyton

Table 4

Occurrence (+) of the ciliates found in Lake Qarun (system of Corliss 1979)

	f	ath	th
Class: **Kinetofragminophora**			
Subclass: **Gymnostomata**			
Loxophyllum setigerum	.	.	+
Orthodonella hamatus	.	.	+
Remanella rugosa	.	.	+
Remanella faurei	.	.	+
Class: **Oligohymenophora**			
Subclass: **Hymenostomata**			
Cyclidium citrullus	+	+	+
Frontonia leucas	+	.	+
Pleuronema coronatum	+	.	+
Pleuronema marinum	+	.	+
Uronema filificum	.	+	+
Uronema nigricans	+	+	+
Class: **Polyhymenophora**			
Subclass: **Spirotricha**			
Order: **Heterotrichida**			
Condylostoma patulum	.	.	+
Condylostoma reichi	.	+	+
Strombidium kahli	.	+	+
Order: **Hypotrichida**			
Aspidisca aculeata	.	.	+
Aspidisca pulcherrima	.	.	+
Diophrys scutum	.	.	+
Epiclintes ambiguus	.	.	+
Euplotes charon	.	.	+

Abbreviations: f - freshwater, ath - athalassic saline, th - thalassic saline

Table 5

Occurrence (+) of the ciliates found in the Coorong (system of Corliss 1979)

	f	ath	th	e	m
Class: **Kinetofragminophora**					
Subclass: **Gymnostomata**					
Holophrya simplex	.	.	+	.	.
Spathidium muscicola	.	.	.	.	+
Subclass**: Vestibulifera**					
Sagittaria australis	.	.	.	+	.
Class: **Oligohymenophora**					
Subclass: **Hymenostomatida**					
Cinetochilum australis	.	.	.	+	.
Cyclidium sp.	.	.	.	+	.
Homalogastra setosa	.	.	.	.	+
Pseudocohnilembus persalinus	.	.	+	.	.
Uronema nigricans	+	+	+	.	.
Class: **Polyhymenophora**					
Subclass: **Spirotricha**					
Order: **Heterotrichida**					
Blepharisma sp.	.	.	.	+	.
Order**: Hypotrichida**					
Amphisiella sp.	.	.	.	+	.
Cladotricha halophila sp. n.	.	.	.	+	.
Cladotricha edaphoni sp. n.	.	.	.	+	.
Gonostomum affine	.	.	.	+	.
Oxytricha longa	.	.	.	+	.
Paragastrostyla sp.	.	.	.	+	.
Urosomoida agilis	.	.	.	+	.
Urosamoida sp.	.	.	.	+	.

Abbreviations: f - freshwater, ath - athalassic saline, th - thalassic saline, e - edaphon, m - moss

Table 6

Biometric characterization of *Spathidium macrostomum* sp. n. (in µm)

Character	Min	Max	x	SD	Sx	n
Body length	110	150	128.33	15.41	5.13	11
Body width	40	80	52.50	13.6	4.29	11
Length of oral slit	80	110	93.63	8.96	2.70	11

Abbreviations: n - sample size, SD - standard deviation, Sx - standard error of the mean, x - arithmetic mean

Table 7

Biometric characterization of four *Cyclidium glaucoma* populations based on wet silver-impregnated specimens (in µm)

	Berger and Thompson (1960) Champaign -Urbana	Blacker (1975) Death Valley	Didier and Wilbert (1981) Bonn	Saskatchewan
Character				
Body length x width	20.3 x 11.7	20.7 x 10.5	16.5 x 7.6	24.7 x 19.8
Kineties	10	10	11	13
Kinetosomes in UM	28-29	27-30	28-34	28-34
Oral ribs	-	9	9-10	7-9
Macronucleus	1	2	1	1
Macronucleus diam.	3.5	3.5	5.45	7.5
Micronucleus	1	?	1	1
UM length:body length	0.59	0.59	0.4	0.51
Kinety fragments in M1	6	7-8	6-7	6-8
Kinetosomes in kinety 1*	14	14-15	11-12	15-18

UM - undulating membranes; *kinetosome pairs counted as a single kinetosome

Table 8

Biometric characterization of *Cyclidium citrullus* based on wet silver-impregnated specimens (in µm). Population of Birket Qarun, Egypt

Character	Min	Max	Me	x	SD	Sx	n
Body, length	21	27	24	24.1	1.75	0.45	20
Body, width	14	18	15	15.7	1.33	0.34	
UM, length	15	19	17	16.9	0.96	0.24	20
Proportion UM length:body length	0.70						
Kinetosomes in UM	*ca* 40						
Oral ribs	5	7	6	6	0.8	0.4	10
Fragments of kineties in M_2	7	9	8	7.8	0.64	0.22	10
Macronucleus	1						
Macronucleus	9	13	10	10.2	1.64	0.73	20
Micronucleus	?						
Kineties	14	16	15	14.8	0.6	0.2	20
kinetosomes in	25	30	27	27.4	1.33	0.44	15
Kinety 1							
paired (above)	10	13	12	11.5	0.88	0.29	
and single	3	5	4	4.3	0.67	0.21	
kinetosomes in	16	19	17	17.42	1.13	0.43	15
Kinety N							
paired (above)	7	8	7	7.28	0.48	0.18	
and single	2	3	3	2.75	0.46	0.16	
kinety N, length	13	15	14	14.0	1.0	0.57	
kinetosomes in	21	24	23	22.87	1.2	0.44	15
Kinety N -1							
paired (above)	7	9	8	7.8	0.64	0.22	
and single	5	8	7	6.9	0.75	0.21	
kinety N -1, length	16	17	17	16.9	0.57	0.33	

Table 8 (cont.)

kinetosomes in Kinety N -2	14	21	19	17.6	2.1	0.97	15
paired (above)	5	7	7	6.4	0.89	0.4	
and single	4	5	5	4.8	0.44	0.2	
Kinety N -2, length	17	19	17	17.75	0.95	0.47	15

Abbreviations: Me - median, n - sample size, SD - standard deviation, Sx - standard error of the mean, x - arithmetic mean, M - membranelle, N - last, left kinetie (kinetie 14), UM - undulating membrane

Table 9

Biometric characterization of *Cladotricha halophila* sp. n. based on protargol impregnated specimens (in µm)

Character	Min	Max	x	SD	Me	n
Body length	102	172	138.4	14.7	138	17
Body width	5	39	30.0	4.0	30	1
AZM length	44	60	54.5	4.4	55	17
Number of AM	24	26	24.3	0.6	24	17
Macronuclear segments	5	14	9.6	2.0	10	17
Ma. length	6	17	12.1	4.0	15	17
Ma. width	6	9	7.0	0.9	7	17
Number of Mi	2	4	2.9	0.6	3	10
Number of FC	8	10	9.0	0.7	9	16
Number of VR 1	27	31	28.5	1.4	28	17
Number of VR 2	11	23	18.7	2.8	19	17
Number of cirri in LMR	19	26	21.6	2.3	21	17
Number of cirri in RMR	26	34	29.5	1.8	30	17
Number of DC	3	3	3	0	3	17
Number of CC	3	3	3	0	3	17

Abbreviations: Me - median, n - sample size, SD - standard deviation, x - arithmetic mean, AM - adoral membranelle, AZM - adoral zone of membranelles, CC - caudal cirri, DC - dorsal cilia, FC - frontal cirri, LMR, RMR - right and left marginal cirri, Ma - macronucleus, Mi - micronucleus, VR - ventral row of cirri

Table 10

Biometric characterization of *Cladotricha edaphoni* sp. n. based on protargol-impregnated specimens (in µm)

Character	Min	Max	x	SD	Me	n
Body length	90	125	114.1	11.9	119	10
Body width	32	49	42.2	6.2	42	10
AZM length	32	41	38.4	2.8	39	10
Number of AM	20	23	21.3	0.9	21	10
Macronuclear segments	15	20	16.8	1.5	16	10
Diam. of Ma	4	4	4	0	4	10
Number of micronuclei	4	4	4	0	4	10
Number of FC	8	9	8.4	0.5	8	10
Number of VR 1	6	11	7.9	1.9	7	10
Number of VR 2	14	22	18.2	3.5	16	10
Number of cirri in LMR	18	23	19.4	1.4	19	10
Number of cirri in RMR	20	30	25.4	3.1	26	10
Number of DC	3	3	3	0	3	10
Number of CC	3	3	3	0	3	10

Abbreviations: Me - median, n - sample size, SD - standard deviation, x - arithmetic mean, AM - adoral membranelle, AZM - adoral zone of membranelles, CC - caudal cirri, DC - dorsal cilia, FC - frontal cirri, LMR, RMR - right and left marginal cirri, Ma - macronucleus, VR - ventral row of cirri

Table 11

Biometric characterization of two *Epiclintes ambiguus* populations: in the Atlantic (upper lines), data from Wicklow and Borror 1990, and in Lake Qarun (lower lines). All data are based on protargol-impregnated, contracted specimens (in µm)

Character		Min	Max	x	SD	Me	n
Body length	Atlantic	170	265	-	-	-	-
	Lake Qarun	66	103	77.7	11.83	76	10
Body width		35	54	-	-	-	-
			34	41	37.8	2.69	3810
Number of AM in AZM		51	71	63	5.0	-	25
			41	56	44.81	4.28	4511
Macronuclear segments		-	-	-	-	-	-
			40	100	56	18.00	5011
Number of BC		1	2	1.5	0.5	-	25
			0	1	0.5	0.5	18
Number of VC		13	15	13.8	0.8	-	25
			8	13	11.44	1.74	129
Number of cirri in MCL		65	93	77.0	7.7	-	25
			45	63	52	6.67	518
Number of cirri in MCR		63	94	82.3	7.2	-	25
			52	72	61.28	7.58	607
Number of DC		3	3	3	0	3	25
			4	4	4	0	47
Number of TC		18	34	30.4	3.4	-	25
			20	29	24.62	3.37	248

Abbreviations: Me - median, n - sample size, SD - standard deviation, x - arithmetic mean, AM - adoral membranelle, AZM - adoral zone of membranelts

Table 12

Biometric characterization of *Euplotes pterotae* sp. n. All data are based on wet silver impregnated specimens (in µm)

Character	Min	Max	x	SD	Me	n
Body length	95	193	144	11.0	140	24
Body width	60	138	98	9.9	94	24
AZM length	72	120	93	3.57	95	19
Number of AM	56	72	64	5.1	65	23
Number of Mi	1	1	1	0	1	24
Number of FC	10	10	10	0	10	20
Number of TC	5	5	5	0	5	24
Number of CC	5	7	5.8	0.59	5	41
Number of DC	10	11	10.1	0.3	10	20
Number of DC cilia/DC	17	25	20.9	2.3	21	20

Abbreviations: Me - median, n - sample size, SD - standard deviation, x - arithmetic mean, AM - adoral membranelle, AZM - adoral zone of membranelles, CC - caudal cirri, DC - dorsal cilia, FC - frontal cirri, TC - transverse cirri

Table 13

Biometric characterization of *Uronychia magna*. All data based on protargol-impregnated specimens (in µm)

Character	Min	Max	x	SD	Me	n
Body length	210	313	252	31.32	252	14
Body width	118	243	197.5	34.3	200	14
AM in AZM 1	10	12	10.9	0.5	11	17
AM in AZM 2	4	5	4.1	0.3	4	17
Macronuclear segments	12	20	15.9	2.1	15	17
Number of micronuclei	1	6	1.8	1.6	1	9
Number of FC	4	5	4.6	0.5	4	10
Number of TC	5	5	5	0	5	17
Number of DC	6	6	6	0	6	17
Number of DC cilia/DC	190	230	-	-	-	-
Number of CC	3	3	3	0	3	17

Abbreviations: Me - median, n - sample size, SD - standard deviation, x - arithmetic mean, AM - adoral membranelle, AZM - adoral zone of membranelles, CC - caudal cirri, DC - dorsal cilia, FC - frontal cirri, TC - transverse cirri

Table 14

Ratio of the number of freshwater and euryhaline species (x) to that of thalassohaline and athalassohaline species (h) in waters of the indicated salinity

Salinity, ‰	3.73	4.32	23.9	24.8*	25.37	27.08	53.21	95.92	370.2
x : h	0	0	0.6	0.46	1.5	1.33	0.75	0.57	0.6

*Lake Qarun

C. citrullus, so that nothing can be said about variation in this species.

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Trimyema shoalsia sp. n., an Anaerobic, Microaerotolerant Marine Ciliate from Appledore Island, Gulf of Maine, USA

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Summary. A new species of *Trimyema* was discovered from a sediment sample from the intertidal zone of Appledore Island, Gulf of Maine, USA. Its morphology was studied using live and protargol stained specimens. The new species is the smallest reported, measuring 13.4-23.7 x 9.3-17.5 µm, and has the fewest somatic kineties (14-24). A characteristic grouping of kinetosomes, termed the epaulet, occurs on the right lateral dorsal surface or "shoulder" of the cell. The oral architecture is distinct from that reported for other species. This paper details the morphological characters that must be examined in order to provide thorough descriptions and ensure proper identifications of *Trimyema* species: cellular and macronuclear length and width, number of vestibulary ciliary rows, number of somatic spirals, length of vestibulum, distance between posterior end of body and posterior end of ciliary spirals, cell shape, number of somatic kineties, and position of epaulet.

Key words. Cultivation, cryopreservation, morphology, taxonomy, Trimyemidae, *Trimyema shoalsia* sp. n.

INTRODUCTION

Trimyema is generally found in organically enriched environments; *T. compressa*, the type species, was originally observed in sewage Imhoff tanks (Lackey 1925). Goosen et al. (1990) concluded that *T. compressa* was an anaerobic, microaerotolerant ciliate.

Although *T. compressa* was found originally in freshwater, other species in the genus have most often been reported from marine environments (Kahl 1928; Fauré-Fremiet 1962; Tucolesco 1962a, b; Jankowski 1964; Borror 1972; Grolière et al. 1980). Two species, *T. compressa* and *T. minuta*, are apparently euryhaline (Kahl 1926, 1928; Fauré-Fremiet 1962; Tucolesco 1962b).

There are eight species of *Trimyema*: *T. alfredkahli*, *T. claviformis*, *T. compressa* (type species), *T. echinometrae*, *T. kahli*, *T. marina*, *T. minuta*, and *T. pleurispiralis* (Augustin et al. 1987). Most species descriptions are based solely upon live material and lack key morphological characters revealed by silver staining. Typically, size, shape, and number of somatic spirals have been used to separate species. Oral structure has been ignored or poorly documented and is known only for *T. compressa*, *T. echinometrae* and *T. pleurispiralis* (Borror 1972, Grolière et al. 1980, Detcheva et al. 1981, Augustin et al. 1987). Additional problems arise from confusion relating to the orientation of the cell, resulting in poor descriptions and inverted images (Augustin et. al. 1987).

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Trimyema compressa is the best characterised species of the genus. The first thorough description was given by Augustin et al. 1987. However, there is considerable morphological variability in strains that have been assigned to this species (compare Augustin et al. 1987, Serrano et al. 1988).

Stomatogenesis has been studied only in *T. compressa* (Serrano et al. 1988). However, the micrographs, morphometric data, and illustrations indicate that the strain studied was a species distinct from *T. compressa* sensu Augustin et al. (1987).

MATERIALS AND METHODS

Isolation and culture

Isolates of the ciliate were obtained from intertidal zone sediments entrapped in a rock crevice adjacent to the cliffs on the south shore of Appledore Island, Maine, USA. Material from the bottom of this crevice was collected with a sterile 2 ml pipette attached to a 10 ml syringe by a length of rubber tubing. Sub-samples were placed in 16 x 125 mm glass screw-capped test tubes containing 12 ml of bacterized American Type Culture Collection (ATCC) medium 1525 (Nerad 1993). The medium was bacterized by inoculating with *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC 27889) at least 24 h previously. Upon arrival at ATCC, the cultures were placed at 25°C and monitored daily. When the ciliate was observed in suitable numbers (approx. 1 x 10^5 cells/ml), 0.25 ml aliquots were transferred to the following media: 12 ml of bacterized ATCC medium 1525 in glass 16 x 125 mm screw-capped test tubes (microaerobic condition) and 10 ml of bacterized ATCC medium 1525 in T-25 tissue culture flasks (aerobic condition).

Single cells were isolated using the method of Molina and Nerad (1992). The cultures were maintained in 12 ml aliquots of bacterized ATCC medium 1525 in 16 x 125 ml screw-capped test tubes. Microaerobic conditions were maintained by screwing test tube caps on tightly. The cultures were incubated on a 5-15° horizontal slant at 25°C. Sub-culturing was accomplished by gently inverting the test tube culture five times and transferring a 0.2-0.25 ml aliquot to a freshly bacterized tube of medium.

Cryopreservation

Twenty test tubes, each with 12 ml of bacterized ATCC medium 1525, were inoculated with 0.25 ml of a growing culture of *Trimyema shoalsia* sp. n. and cultivated as above. Five days post-inoculation, the cultures were inverted several times, transferred to plastic centrifuge tubes, and centrifuged at 410 x g for 5 min. The volume of the supernatant in each tube was reduced to approximately 0.5 ml. Approximately 10 ml of the supernatant was reserved for preparation of the cryoprotective solution. The cells were resuspended, pooled in one tube, and again centrifuged as above. The cell pellet was resuspended to a final volume of 5.5 ml. The reserved supernatant was used to make an 18% (v/v) dimethyl sulfoxide (DMSO) cryoprotective solution. The solution was prepared as follows: 1.8 ml of DMSO was allowed to solidify in a iced glass test tube, 8.2 ml of supernatant was added, and the tube inverted until the DMSO was brought into solution. A volume of cryoprotective solution equal to the volume of the concentrated cell suspension was added stepwise in three equal aliquots at 2 min intervals. The cell suspension was then aliquoted in 0.5 ml amounts to 1.2 ml plastic, conical bottom cryo-tubes (Nunc, Denmark). The cells were allowed to equilibrate for 15 min at room temperature and then frozen in a Cryo-Med 1010 controlled rate freezing unit (Cryo-Med Inc., Mt. Pleasant, MI) using the triphasic cooling cycle described in Nerad and Daggett (1992). Frozen ampoules were stored in liquid nitrogen storage tanks at temperatures below -150 °C.

Light microscopy

Live and fixed material were observed and measured with a Zeiss Axioskop fitted with phase contrast elements. Photographs were taken with a Zeiss MC 80 system using Tech Pan film. Drawings were done to scale (SAS).

A clonal population was studied using both live and fixed material. Cells for fixation were prepared by centrifuging at 230 x g for five minutes. The supernatant was removed and cells resuspended to a final volume of 0.5 ml. Resuspended cells were then added to 17.5 ml of sterile ATCC medium 1525. Two ml of Bouin's fluid (Lee et al. 1985) was immediately injected into the sample. Fixed material was stained with the quantitative protargol procedure of Lynn (1992) and used for morphological data.

Live cells were observed by placing a single drop of concentrated cells on a clean, degreased slide and covering with a coverslip. Excess medium was removed with a tissue and the edges of the coverslip sealed with petroleum jelly (Vaseline brand). Cells were observable up to four hours with little effect from evaporation.

The morphological characterization of the ciliate is solely the work of T. A. Nerad and S. A. Schaffer, who accept full responsibility for the identification of the species.

RESULTS

Cultivation

Growth of *Trimyema shoalsia* sp. n. could only be sustained under microaerobic conditions. The ciliate could not be propagated aerobically.

Description of *Trimyema shoalsia* sp. Nerad et Schaffer, 1994

Parenthetical values are as follows: standard deviation, range, and sample size. The length of Bouin's fixed, protargol stained specimens averaged 18.5 µm (± 2.4µm, 13.4-23.7 µm, n=30), with an average width of 13.4 µm (± 1.8 µm, 9.3-17.5 µm, n=30) (Table 1). Stained cells were egg-shaped with a single long caudal cilium (Figs. 1A-C, 7). Live material was observed to have two forms. Well fed cells were egg-shaped and similar in appearance to stained material although slightly longer and wider. Starved cells were observed to be laterally compressed. The macronucleus was spherical to slightly ovate (Figs. 6, 7) with

Table 1

Summary of morphometric data for *Trimyema* species based on original descriptions unless noted. All measurements in µm

Species	Cell length	Cell width	Mac. length	Mac. width	Vestibulary ciliary rows	Number of somatic spirals	Number of caudal cilia	Length of vestibulum: longitudinal axis	Distance between posterior end of body and posterior end of ciliary spirals	Cell shape	Number of somatic kineties	Position of epaulet
T. shoalsia sp. n.	18.5 13.4-23.7	13.4 9.3-17.5	5.3 4.1-6.2	5.4 4.1-6.2	2.0	3.0	1.0	4.9 3.1-6.2	8.9 5.2-14.4	egg shaped	21 14-26	kineties 3, 4, 5
T. compressa[1]	39.05 32-47	22.3 17-26	11.05 9-14	9.35 6-12	3.0	3.0	1.0	9.8 7-15	11.0 7-15	fusiform to plum S shape	NR 50-60	kineties[2] 3, 4, 5
T. echinometrae	31.0 27-40	17.0 13-20	NR 5-7.5	NR NR	3.0	7.0	1.0	NR NR	NR NR	"top like"	NR 60-70	NR
T. pleurispiralis	NR 16-23	NR 16-23[3]	NR NR	NR NR	2.0	4-6	1.0	NR NR	NR NR	egg like	NR NR	NR
T. alfredkahli	60 NR	NR NR	NR NR	NR NR	NR	NR	1.0	NR	NR NR	oblong, slender tapered ant to posterior	NR NR	NR
T. claviformis	40 NR	NR NR	NR NR	NR NR	NR	NR	1.0	NR NR	NR NR	club shaped	NR NR	NR
T. kahli	NR 36-40	NR NR	NR NR	NR NR	NR	NR	1.0	NR NR	NR NR	inverted S shape	NR NR	NR
T. marina	40 NR	NR NR	NR NR	NR NR	NR	5-6[4]	1.0	NR NR	NR NR	slender, fusiformg to oblong	NR NR	NR
T. minuta	20 NR	NR NR	NR NR	NR NR	NR	NR	1.0	NR NR	NR NR	beak like pharynx	NR NR	NR

Abbreviations: NR - Not recorded in original description, [1]from Augustin et al. 1987, [2]reported by Schmall, 1976 only, [3]usually less than 20µm , [4]originally depicted with 3 ciliary spirals

an average length of 5.3 µm (± 0.59 µm, 4.1-6.2 µm, n=30). The width averaged 5.4 µm (± 0.76 µm, 4.1-6.2 µm, n=30). The macronucleus was located in the center of the cell and strongly argentophilic for most specimens (Figs. 3, 6, 7). No rod-like structures were observed to be associated with the macronucleus. The micronucleus was spherical and usually well stained (Fig. 7). It averaged 1.0 µm in diameter (± 0.0 µm, 1.0-1.0 µm, n=30) and was proximal to the macronucleus.

Thin argentophilic lines, presumed to be basal microtubules, paralleled the somatic kineties (Figs. 1A-C, 2, 3). Kinetosomes on the ventral surface were located just to the left of these lines, occasionally appearing to be directly on the lines (Figs. 1B, 2). An average of 21 somatic kineties (± 2.3, 14-26, n=30), composed of 3-4 single kinetosomes, ran longitudinally and were ciliated. The kinetosomes had the appearance of being organized into three oblique spirals or paratenes (Figs. 1A-C, 2). Somatic spirals for *T. shoalsia* sp. n. began on the right margin of the ventral surface (Fig. 2), continued on the dorsal surface greater than 1-1/4 turns, and ended. The posterior third of the ciliate was unciliated except for occasional scattered cilia usually appearing on the ventral surface and the caudal cilium (Fig. 7). The average distance from the posteriad kinetosome of the last somatic spiral to the posterior of the cell was 8.9 µm (± 2.19 µm, 5.2-14.4 µm, n=30). Mucocysts were not observed.

There were three longitudinal files of kinetosomes on the right lateral dorsal surface of the cell. This assembly of kineties was designated the epaulet (Figs. 1A, 3, 4) and was comprised of a total of 17 kinetosomes. The first and second kinetosomes of kinety 3 and the first kinetosomes of kineties 4-5 were paired anteriorly. Therefore, kinety 3 had 5 kinetosomes and kineties 4 and 5 had 6 kinetosomes (Figs. 1A, C, 3). The additional kinetosomes in kineties 3-5 appear to be unciliated in stained material (Figs. 1A, 3).

A file of three kinetosomes, referred to henceforth as the ventro-lateral fragment (VLF), was observed on the right ventro-lateral surface. The VLF was arranged as a pair of kinetosomes separated from a single kinetosome and oriented at a 45° angle to longitudinal axis of the cell (Figs. 1B, 2, 4).

The oral region is ovoid and the length of its longitudinal axis is approximately one-third of the body length (Figs. 1C, 4, 5). The average distance from the apex of the vestibular opening to its posterior aspect was 4.9 µm(± 2.2 µm, 3.1-6.2 µm, n=30). The oral structure is composed of two ciliary rows which are arranged as a semi-circle on the left margin of the oral region (Figs. 1D, 4, 5). The outer oral kinety (V1) begins at the 11 o'clock position and continues posteriad along the edge of the oral region to the bottom of the oral region (6 o'clock position) (Figs. 1D, 5). The inner oral kinety (V2) begins at the one to 2 o'clock position and continues posteriad to approximately the 6 o'clock position (Figs. 1D, 4). Both oral kineties are composed of single kinetosomes. At the beginning of V1 a group of six pairs of ciliated kinetosomes, doublets, form a rosette-like pattern (Figs. 1D, 3-5). A well stained cytopharynx extended approximately two-thirds the length of the cell (Fig. 6).

Type locality

The ciliate was collected from the intertidal zone adjacent to the cliffs on the south shore of Appledore Island, Maine during August, 1993. Appledore Island is a member of a cluster of islands compromising the Isles of Shoals in the Gulf of Maine. *Fucus* sp., as well as other marine plants, were attached to the rocky substrate. The sample was taken from the sediment trapped in the rocks at low tide. The presence of H_2S was noted by the grey to black color of the sediments and by its rotten egg odor.

Type specimen

One protargol stained slide has been deposited as the syntype in the International Protozoan Type Slide Collection, National Museum of Natural History, Smithsonian Institution, Washington, D.C., U.S.A USNM # 43220. The ciliate has been deposited at the American Type Culture Collection and assigned accession number 50416. The paratype has been placed in the collection of the junior author (SAS) as part of a collection documenting protists from the Isles of Shoals, Gulf of Maine.

Etymology

The ciliate is named for the cluster of islands compromising the Isles of Shoals, Gulf of Maine. Following the conclusion reached by Augustin et al. (1987), we are treating the generic name as being of the feminine gender.

Diagnosis

Trophont length 13.4-23.7 µm (average 18.5 µm), width 9.3-17.5 µm (average 5.4 µm). Stained cells egg-shaped. Live cells with two forms: well fed cells egg-shaped, starved cells compressed laterally. Macronucleus spherical to oval, located in the center third of

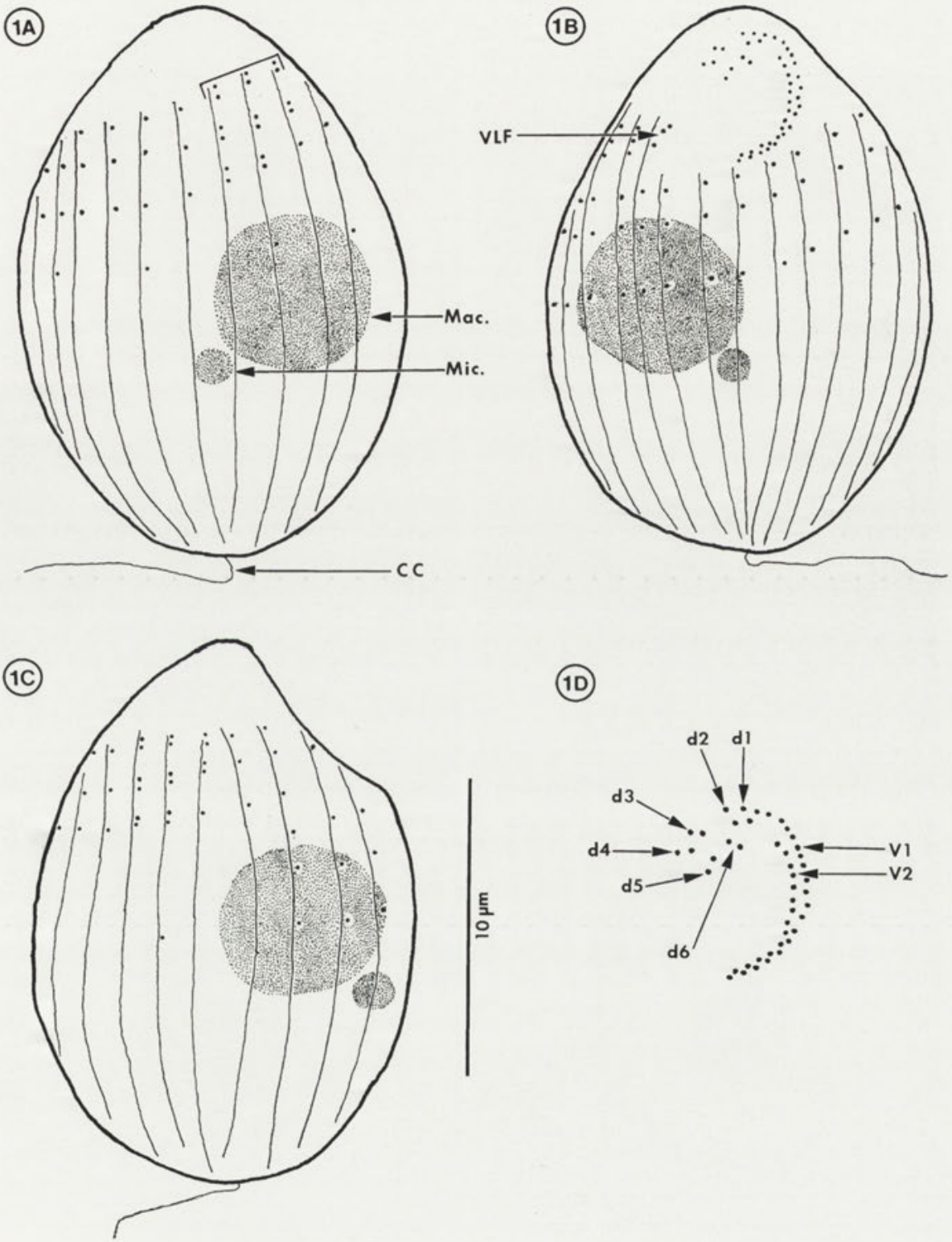
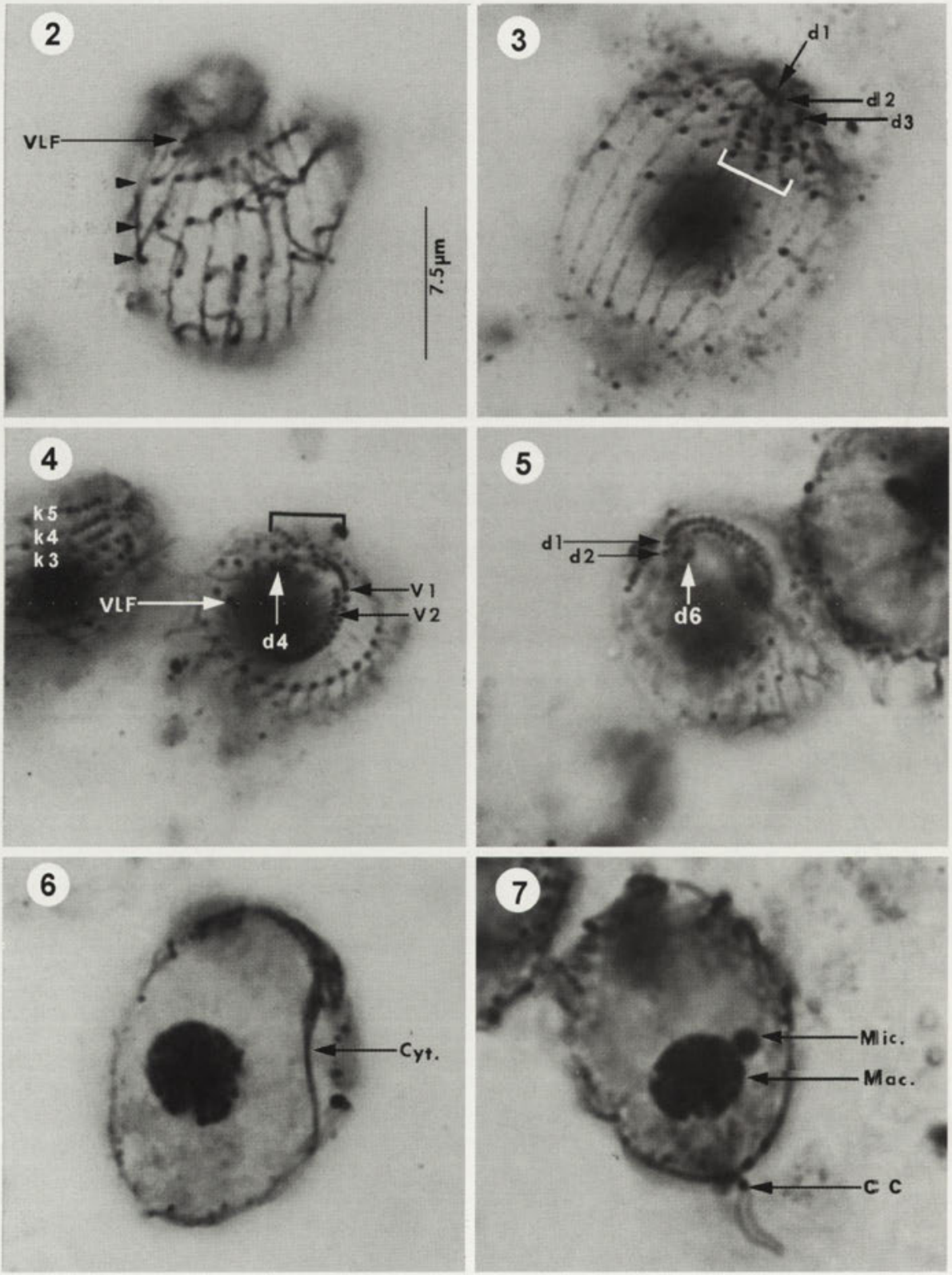


Fig. 1. Scaled line drawings of protargol stained specimens of *Trimyema shoalsia* sp. n. A - dorsal view indicating epaulet (bracket), macronucleus (Mac.), micronucleus (Mic.), and caudal cilium (CC). B - ventral view indicating somatic spirals, argentophilic lines, and ventro-lateral fragment (VLF). C - right lateral view of cell indicating relative position of somatic spirals. D - oral detail showing outer oral kinety (V1), inner oral kinety (V2), and doublets (d1-d6). Bar - 10 µm (1A-1C)



Figs. 2-7. Micrographs of protargol stained specimens *Trimyema shoalsia* sp. n. (x 1000). 2 - ventral view indicating argentophilic lines, ventro-lateral fragment (VLF) and the somatic spirals (arrow heads). 3 - dorsal view detailing the epaulet (bracket), and doublets d1-d3. 4 - apical view of cell depicting epaulet on right dorso-lateral surface (bracket), outer (V1) and inner (V2) oral kineties, doublet 4 (d4), ventro-lateral fragment (VLF) and kineties 3, 4, 5 (k3-k5). 5 - oral detail indicting doublets d1, d2 and d6. 6 - cytopharynx stained by the protargol method. 7 - macronucleus (Mac.), micronucleus (Mic.) and caudal cilium (CC). Bar - 7.5 µm (2-7)

cell. Fourteen (14) to 26 somatic kineties. Epaulet on right dorso-lateral surface, contains kinetosomes from kineties 3, 4, and 5. Posterior third of cell unciliated except for occasional scattered kinetosomes and caudal cilium. No mucocysts. Oral region ovoid; longitudinal axis 3.1-6.2 µm (average 4.9 µm). Two ciliary rows arranged in semi-circle at the entrance of the oral region, meeting at the posterior only. Group of six pairs of kinetosomes at the anterior of V1.

DISCUSSION

Trimyema shoalsia sp. n. shares the following generic characters with the type species: (1) Oral region and cytostome located at the anterior end. (2) Somatic ciliature as short longitudinal kineties arranged to appear as three spirals. (3) Single caudal cilium present. (4) Oral ciliature arranged as a semicircle at the left margin of the oral region. (5) Longitudinal lines paralleling kineties.

Species may be separated into two groups based on the numbers of spiraling somatic kineties (Augustin et al. 1987). *Trimyema compressa* (freshwater and marine) and *T. minuta* (freshwater and marine) each have three somatic ciliary spirals. Species with more than 3 ciliary spirals are: *T. alfredkahli* (marine), *T. claviformis* (marine), *T. kahli* (para-marine), *T. marina* (marine), *T. pleurispiralis* (marine), and *T. echinometrae* (endocommensal of sea urchin). Based on gross morphology and habitat, the two most closely related species are *T. compressa* and *T. minuta*.

Morphologically, *T. shoalsia* sp. n. is a smaller ciliate than *T. compressa* for all characters examined. *T. compressa* has been reported to have three "vestibular" kineties (here, oral kineties); two kineties were observed in *T. shoalsia* sp. n. The presence of two kineties in the "vestibulum" (here, oral region) was consistent for all specimens examined (n=30). Borror (1972) reported that *T. pleurispiralis* had only an outer and inner "polykinety". Augustin et al. (1987) included three "vestibular" kineties as a character of the family. This character is based on the oral architecture of *T. compressa* (Augustin et al. 1987) and interpretations by Augustin et al. (1987) of oral structures illustrated in the original descriptions of *T. pleurispiralis* (Borror 1972) and *T. echinometrae* (Grolière et al. 1980). Based on our reviews of the literature and this study, we suggest that the familial character be amended from three to two-three oral kineties aligned on the left margin of the oral region.

The oral architecture of *T. compressa* is distinctive from that of *T. shoalsia* sp. n. In *T. compressa*, the files of oral kinety one and two are nearly aligned at the posterior position (Augustin et al. 1987). Kinety one begins at the 10 o'clock position and ends posteriad at the 6 o'clock position, kinety two begins at the 11 o'clock position and ends posteriad at the 6 o'clock position. Augustin et al., reported five tetrads of kinetosomes (possibly pairs of kinetosomes with associated parasomal sacs) at the anterior of the "vestibular" ciliature (1987). The tetrads were arranged in a C-shaped pattern. In *T. shoalsia* sp. n., the oral kineties overlap in the same general position, the region from 1 to 2 o'clock posteriorad to the 6 o'clock position. The first two pairs of kinetosomes at the anterior of V1 appear to be arranged as a tetrad but the remaining four pairs are separated doublets and are arranged in a rosette-like pattern. Parasomal sacs were not evident.

Schmall (1976) reported a strain of *T. compressa* with an epaulet indistinguishable from that observed in *T. shoalsia* sp. n. Augustin et al. (1987) indicated the presence of an epaulet but it is not clearly evident in their photomicrographs. Serrano et al. (1988) make no mention of an epaulet. The epaulet may be sensory in function, similar to the clavate cilia of *Didinium* (Corliss 1979). Clavate cilia lack the central pairs of microtubules in the axoneme (Corliss and Lom 1985). Ultrastructural studies are required to determine if the central pairs are lacking, thus giving further evidence for a sensory function. The presence of an epaulet may be of value in separating species.

Trimyema compressa has been reported to have 50-60 somatic kineties (Augustin et al. 1987, Serrano et al. 1988). *Trimyema shoalsia* sp. n. has 14-26 kineties. The distance from the lowest kinetosome to the posterior of the cell is a character which may be species specific. This distance for *T. compressa* is approximately 1/4 of the cell length, i.e., the anterior 3/4 of the cell is covered with spiraling cilia (Augustin et al. 1987). The distance for *T. shoalsia* sp. n. is approximately 1/2 the cell length, i.e., the anterior 1/2 of the cell is covered with spiraling cilia.

The VLF has been observed by other researchers in *T. compressa* (Augustin et al. 1987, Serrano et al. 1988). Augustin et al. (1987) illustrated a group of 4 kinetosomes located mid-ventrally, three aligned in a file at a 45° angle to the longitudinal axis of the cell. The posterior most kinetosome of this fragment was sharply out of alignment with the preceding kinetosomes. The arrangement of the kinetosomes in this file is very

similar to that observed in *T. shoalsia* sp. n. Serrano et al. (1988) observed a fragment close to the base of the oral region, at the mid-ventral position, and parallel to the longitudinal axis of the cell, which they termed kinety *n*. We consider kinety *n* to be homologous to the VLF. The VLF of *T. shoalsia* sp. n. is distinct from both descriptions of this fragment; being further removed from the oral region, having smaller numbers of kinetosomes, and in shape.

The orientation of the somatic spirals clearly separates *T. shoalsia* sp. n. from *T. compressa*. The somatic spirals for *T. compressa* begin and end on the right margin of ventral surface, each undergoing a 360° turn. Somatic spirals for *T. shoalsia* sp. n. begin on the right margin on the ventral surface, continue past through 360°, ending on the dorsal surface (a turn of greater than 450°). Curved, rod-shaped, methanogenic bacteria have been seen in *T. compressa* (Augustin et al. 1987, Wagner and Pfennig 1987, Goosen et al. 1990). These structures were not observed in protargol stained specimens of *T. shoalsia* sp. n. Oval-shaped granules, probably mucocysts, observed in protargol preparations of *T. compressa* (Augustin et al. 1987) were absent from *T. shoalsia* sp. n.

The only other species of *Trimyema* with three somatic ciliary spirals is *T. minuta*. This is a small species, approximately 20 µm in length, with a "beak-like vestibulum". A ridge or keel extends from the "vestibulum" dorsally to the posterior (Augustin et al. 1987). This species has been observed infrequently (Wenzel 1961, Tucolesco 1962b), since first observed by Kahl (1931). *Trimyema shoalsia* sp. n. is clearly different from *T. minuta* as it lacks the "beaked vestibulum" and the dorsal keel.

Strains identified as *T. compressa* in the literature exhibit high variability (compare Augustin et al. 1987, Serrano et al. 1988). Serrano et al. (1988) described morphogenesis in a species identified as *T. compressa*, an identification they stated that, " .. agrees with the redescription of Augustin et al". Based on their micrographs, morphometric data, and illustrations, it most likely is a distinct species. The oral architecture differs from the redescribed species, most notably by the presence of two (rarely three) isolated groups of kinetosomes at the anterior of V1, each composed of 6 kinetosomes arranged in two rows of three (Serrano et al. 1988). Augustin et al. observed five tetrads of kinetosomes or pairs of kinetosomes with associated parasomal sacs at the anterior of the "vestibular" ciliature (1987). Secondly, Serrano et al. (1988) reported a ventral file of kinetosomes below the "vestibulum", designated kinety *n*, composed of four to six kinetosomes. This file ran parallel to the longitudinal axis of the "vestibulum", from the beginning of somatic spiral two to the end of somatic spiral one. *T. compressa*, as reported by Augustin et al. (1987), has four kinetosomes, three in a row running at a 45° angle to the longitudinal axis of the vestibulum and may be homologous to kinety *n* of Serrano. However, these kinetosomes are not closely associated with somatic spirals one and two.

Finally, the size range given for length and width of the cells (Serrano et al. 1988) are clearly outside the range given by Augustin et al. (1987). Table 2 summarizes these observations. Detailed study of microscope slides used by Serrano et al. (1988) is required to decide the question of whether those workers were investigating a *Trimyema* species separate from all others in the genus.

Taxonomically, the position of *Trimyema* itself is uncertain. Kudo included the genus in the order Trichostomatida, Butschli, 1889, family Trimyemidae Kahl, 1933 (Kudo 1966). This was later accepted by Corliss (1979) and Curds (1982). Detcheva et al. (1981) placed the genus in the order Vestibulifera in the class Litos-

Table 2

Comparison of morphometric data for strains identified as *Trimyema compressa*. All measurements in µm

Author	Cell length	Cell width	Ant. kin.	Vent. kin.	Stain
Augustin et al. 1987	39.05 32-47	22.3 17-25	5 groups of 4 kinetosomes	3-4 kinetosomes at 45o to longitudinal axis	Protargol
Serrano et al. 1988	65.9 53.9-80.4	54.6 42.3-64.5	2 groups of 6 kinetosomes	6 kinetosomes parallel to to longitudinal axis	Silver carbonate

Abbreviations: Ant. Kin. - Kinetosomal grouping at the anterior of V1, Vent. Kin. - Mid-ventral kinety

tomatea based upon electron microscopic studies. However, Serrano et al. (1988) suggested that *Trimyema* might belong in the class Protostomatea based on their interpretation of their stomatogenic study.

Our interpretation of the ultrastructural study of Detcheva et al. (1981) indicates that *Trimyema* is not a member of the Litostomatea as defined ultrastructurally by various authors (Lynn and Nichols 1985; Leipe and Hausmann 1989; Lipscomb and Riordan 1990, 1992). The ultrastructurally defined somatic monokinetid of litostomes have two sets of transverse microtubules, one radial and the other tangential. Furthermore, the postciliary microtubules insert below the inner alveolar membrane in a characteristic four over three configuration. At their site of origin, microtubular set number nine, the postciliary microtubules are convergent rather than divergent. Additionally, a well developed tela corticalis is present in the litostomes. These characteristic features are not found in the published micrographs of Detcheva et al. (1981) and only one far removed ribbon of what is referred to as transverse microtubules can be seen (e.g. Figs. 3, 4). The postciliary microtubular ribbon is composed of 10-14 microtubules (Detcheva et al. 1981), which differs from the defined seven (three over four) microtubules for litostomes (Leipe and Hausmann 1989).

In an unpublished ultrastructural study of *Trimyema* (Bardele, personal comm.), the single transverse microtubular ribbon appears to be radially oriented. Therefore, the correct systematic placement, based on ultrastructure and using the system of Small and Lynn (1985), would place *Trimyema* within either the Prostomatea or the Oligohymenophorea.

Although Serrano et al. (1988) suggest that *Trimyema* may be a prostome, their study of stomatogenesis is inconclusive. It is impossible to unambiguously interpret all of the steps of stomatogenesis as shown in their figures. Clearly it is of interest to note that oral kinetid fragments figured by Augustin et al. (1987) and discussed as possible oral dikinetids are figured as monokinetids by Serrano et al. (1987). Perhaps the two groups of researchers are looking at different species as has been suggested above. The following characters are not like those of other prostomateans during stomatogensis, while it is true that stomatogensis is indeed ventral as established by Serrano et al. (1988). If there was a true circumoral dikinetid, it would have its postciliary microtubules oriented towards the cytostome. The circumoral dkinetid would also make a complete circuit on the ventral surface and cytokinesis would cleave the proter from the opisthe so that the whole circumoral structure would remain ventral until cytokinesis was completed. Only at the close of this unusual and unequal cleavage would the circumoral oral dikinetid be seen to enclose the whole anterior end of the cell. The paper of Serrano et al. (1988) has only one figure of cytokinesis and this figure appears to be concentric in its constriction of the proter from the opisthe.

Since the current evidence does not support the placement of *Trimyema* in either the class Litostomatea or Protostomatea, as an alternative suggestion it is proposed that the genus may belong in the class Oligohymenophorea. Only a detailed transmission electron microscopic study of the "circumoral dikinetid" during stomatogenesis would allow the correct systematic assignment at the ordinal/class level. Molecular studies may also be required to resolve this taxonomic problem.

In an effort to aid in the description of new and redescriptions of previously characterized species of *Trimyema*, it is proposed that the following guidelines be used: (1) Live and fixed specimens must be studied to obtain an adequate description. (2) Oral structure is of great importance and slight variations may be species specific. Oral morphology is best demonstrated in stained material. (3) Characteristics used for the description of species should include, but not be limited to: cell shape, cell length and width, macronucleus length and width, number of vestibulary ciliary rows, number of somatic spirals, number of kineties, caudal cilia (presence or absence), distance from lowest kinetosome of the somatic ciliary spirals to posterior of cell, epaulets (presence or absence), number and position of kinetosomes in epaulet (if present), and the length of the longitudinal axis of the vestibulum.

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Two New Isosporan Parasites (Apicomplexa: Eimeriidae) from the South American Woodcreeper *Dendrocolaptes certhia*

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Summary. Two new species of *Isospora* are reported in the faecal contents of the barred woodcreeper, *Dendrocolaptes certhia* from Ecuador. Oocysts of *Isospora magna* sp. n. are reported from 1/4 barred woodcreepers. Sporulated oocysts are ovoidal, 29.7 x 24.9 (26-31 x 23-26) µm, with a smooth, colorless, bilayered wall; average shape index is 1.20. No micropyle or oocyst residuum are present, but the oocyst contains 1 large, rough polar granule. Sporocysts are ovoidal, 15.8 x 12.6 (15-20 x 11-14) µm; average shape index is 1.28 with a smooth, single layered wall. The Stieda body is broad dome-like and the substieda body is inconspicuous, with a wavy lower surface and located directly below and slightly to the side of the sporocyst vertical axis. Each sporocyst contains a residuum of fine, uniform granules in a consolidated, lobate cluster. Sporozoites are sausage-shaped with an ovoid, posterior, refractile body, a slightly smaller anterior, refractile body; nucleus is spherical, less distinct and centrally located. *Isospora concentrica* sp. n. is reported from 3/6 barred woodcreepers. Sporulated oocysts are ovoidal, 26.6 x 22.7 (24-30 x 21-25) µm, colorless, with a smooth bilayered wall; shape index is 1.2. No micropyle or oocyst residuum are present, but the oocyst contains a single, ovoid polar body or occasionally 2 polar bodies. Sporocysts are ovoidal to ellipsoidal, 17.2 x 11 (15-18 x 9-12) µm, with a squared or block-shaped Stieda body and an ovoidal or bubble-shaped substieda body located beneath the Stieda body, usually to the left or right of the sporocyst vertical axis; shape index is 1.6. Sporocyst residuum is compact, usually slightly ovoid and composed of uniform, fine granules. Sporozoites are vermiform and appear randomly arranged in the sporocysts with a round posterior refractile body, a large circular nucleus and the anterior part marked with concentric lines.

Key words. *Isospora magna* sp. n., *Isospora concentrica* sp. n., *Dendrocolaptes certhia*, Passeriformes, Dendrocolaptidae, Coccidia, Apicomplexa, Eimeriidae.

INTRODUCTION

Woodcreepers (Order: Passeriformes, Suborder: Tyranni, Family: Dendrocolaptidae) are widespread throughout the Neotropical Realm from Mexico to central Argentina. Dendrocolaptids are called woodcreepers because of their scansorial behavior (Willis 1978). Despite consisting of 13 genera and circa 50 species (Sibley and Monroe 1990), they are a very uniform group in their morphologic structure (Feduccia 1970) differing primarily in preferred foraging substrate as they search for arthropods (Chapman and Rosenberg 1991).

Although woodcreepers are common elements of the avifauna throughout the forested neotropics, no prior

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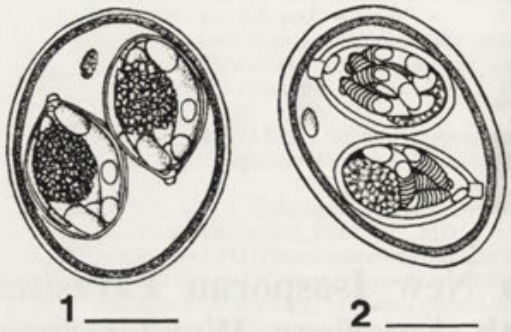
studies have reported coccidian parasites in any of its members. This paper describes 2 new coccidian species found in the barred woodcreeper, *Dendrocolaptes certhia.*

MATERIALS AND METHODS

During bird collecting expeditions to Ecuador in July-August, 1990 and March, 1991, samples were taken from the contents of the small and large intestines of 6 barred woodcreepers and sent to the first author's laboratory for examination. Procedures for preserving faecal material and for measuring and photographing oocysts were as described by McQuistion and Wilson (1989). To eliminate the possibility of spurious oocysts, positive faecal samples with very poor sporulation of the oocysts or faecal samples with fewer than 20 oocysts were not considered for examination of new coccidial species. All measurements are given in µm with size ranges in parentheses following the means. Oocysts were 1-2 months old when examined, measured and photographed.



Figs. 1-2. Composite line drawings of sporulated oocysts of: 1- *Isospora magna* sp. n. 2 - *Isospora concentrica* sp. n. Bars - 10 µm

RESULTS

Isospora magna sp. n. (Figs. 1, 3, 4)

Description of oocysts: oocysts ovoidal, 29.7 x 24.9, (26.0-31.0 x 23.0 - 26.0) (N= 42) with a smooth, bilayered wall ca. 2.0 thick; the inner wall is darker. The shape index (length/width) is 1.20 (1.08 - 1.30). Micropyle and oocyst residuum absent. One subspherical to ovoid, rough polar granule present, about 3.5 in diameter. Sporocysts ovoidal, 15.8 x 12.6 (15.0-20.0 x 11.0-14.0) (N=43); shape index 1.28 (1.07-1.82). The Stieda body is broad, dome-like with an inconspicuous substieda body with a wavy lower surface and located directly below the Stieda body but slightly to the left or right of the vertical axis. Sporocyst residuum composed of uniform, fine granules in a consolidated, lobate, well defined cluster (*ca* 5.5-6.0). Sporozoites sausage-shaped (*ca* 14.0 x 2.5-3.0), with an ovoid, posterior refractile body (*ca* 6.0 x 3.0), a slightly smaller ovoid anterior refractile body (*ca* 5.0 x 2.5) and a round, less distinct, centrally located nucleus. Sporozoites are arranged randomly in sporocyst.

Type-host: *Dendrocolaptes certhia radiolatus* Sclater and Salvin, 1868, (Passeriformes: Dendrocolaptidae). An adult male collected in August 1990 by J. A. Gerwin (JAG #1447) has been deposited at The Academy of Natural Sciences of Philadelphia Museum as ANSP 183223 in April, 1991.

Type specimens: a phototype series and formalin-preserved sporulated oocysts are deposited in the U.S. National Parasite Collection, Beltsville, Maryland 26705, accession no. USNM 83242.

Type location: Ecuador, Provincia de Sucumbios, Imuya Cocha, 0° 34'S, 75° 17' W, 200 m elevation.

Prevalence: 1/2 were infected from Provincia Sucumbios: 0/2 were infected from Provincia de Morona-Santiago, *ca* 5 km SW of Taisha, 2° 22' S, 77o 30' W, 425 m elevation.

Sporulation Time: unknown; oocysts were partially sporulated when received at the laboratory and became fully sporulated after exposed to air for several days prior to examination.

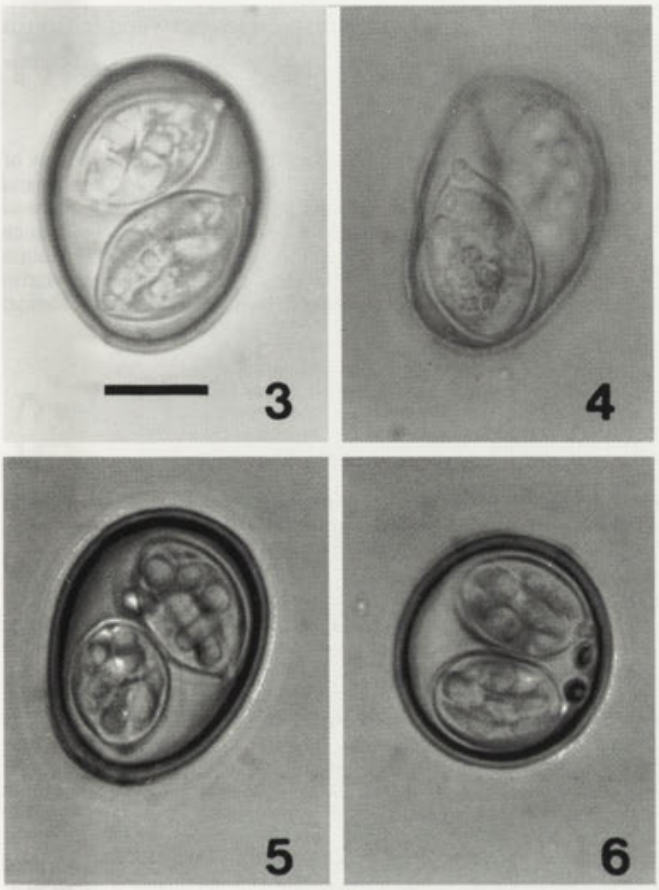
Site of infection: unknown, oocysts found in faeces.

Etymology: the specific epithet, *magna,* is the Latin nominative singular form for large in reference to the overall size of the oocysts.

Remarks: the oocysts are fragile and collapse after 1 hour in saturated sugar solution. In older samples (> 6 months), the sporocyst residuum begins to break up into coarse clumps and is scattered throughout the sporocyst. Although only one bird was found infected with *I. magna,* there were hundreds of oocysts in the sample and all the oocysts had similar morphological characteristics.

Isospora concentrica sp. n. (Figs 2, 5, 6)

Description of oocysts: oocysts ovoid, 26.9 x 22.7 (24-30 x 21-25) (N=41), with smooth, bilayered wall (*ca*



Figs. 3-6. Bright field photomicrographs of sporulated oocysts of: 3-lateral view of *Isospora magna* sp. n. 4 - collapsed oocyst of *Isospora nagna* showing sporocyst with Stieda and substieda body and sporocyst residuum. 5 - lateral view of *Isospora concentrica*. 6 - end view of a *Isospora concentrica* oocyst showing 2 polar bodies. Bar - 10 µm for each figure

2.0 thick) and darker inner layer of approximately equal thickness; shape index (length/width) 1.19 (1.04-1.38). Micropyle and oocyst residuum absent. One, occasionally 2, ovoid polar bodies present. Sporocysts ovoid to ellipsoidal, 17.2 x 11.0 (15.0-18.0 x 9.0-12.0) (N=21), with a squared or block-shaped Stieda body and an ovoidal or bubble-shaped substieda body located beneath the Stieda body, but usually lying to the left or right of the vertical axis; shape index 1.56 (1.42-1.70). Sporocyst residuum compact (*ca* 5.0), usually slightly ovoid and composed of uniform fine granules. Sporozoites are vermiform (*ca* 15-16 x 3), with a round to slightly oblong shaped posterior refractile body, a large circular nucleus and the anterior half marked with concentric lines or grooves. The sporozoites appear randomly arranged around the residuum in the sporocysts.

Type-host: *Dendrocolaptes certhia radiolatus* Sclater and Salvin, 1868, (Passeriformes: Dendrocolaptidae). An adult male collected in August, 1990 by M.B. Robbins (MBR #2972) was deposited in The Academy of Natural Sciences of Philadelphia Museum as ANSP #183221 in April, 1991.

Other host: *Dendrocolapes certhia columbianus* Todd, 1950, (Passeriformes: Dendrocolaptidae), APC #3754, The Academy of Natural Sciences of Philadelphia, ANSP #182407, April, 1991.

Type specimens: a phototype series and formalin-preserved sporulated oocysts are deposited in the U. S. National Parasite Collection, Beltsville, Maryland 26705, accession no. USNM 83243.

Type location: Ecuador, Provincia de Sucumbios, Imuya Cocha, 0° 34' S, 75° 17' N, elevation 200 m.

Other host locations: one *D. c. radiolatus,* APC 3858 was collected from Provincia Morona-Santiago, *ca* 5 km SW of Taisha, 2° 22' S, 77° 30' W, 425 m elevation and *D. c. columbianus,* APC 3754 was collected from Provincia de Esmeraldas, *ca* 20 km NNW of Alto Tambo, 0° 57' N, 58° 33' W, 275 m elevation, Ecuador.

Prevalence: *D. c. radiolatus,* 2/5 were infected with *Isospora concentrica* only; 1/5 were infected with *I. magna* only; *D. c. columbianus* 1/1 was infected with *I. concentrica* only.

Sporulation Time: unknown; oocysts were partially sporulated when received at the laboratory and became fully sporulated after exposed to air for several days prior to examination.

Site of Infection: unknown. Oocysts found in faeces.

Etymology: the specific epithet, *concentrica,* is the Latin nominative singular form for concentric circles in reference to the concentric bands or markings on the anterior end of the sporozoites.

Remarks: the host, *D. c. columbianus,* yielded slightly smaller oocysts 26.6 x 22.7 (24-30 x 21-25) than oocysts from *D. c. radiolatus,* 27.4 x 22.6 (27-28 x 21-24); sporocyst sizes of *D. c. columbianus* 17.1 x 10.9 (16-18 x 10-11) also differed from *D. c. radiolatus* 17.3 x 11.2 (15-18 x 9-12). The oocyst and sporocyst shape indices for the 2 populations of *I. concentrica* are nearly identical; oocyst and sporocyst shape index of *D. c. columbianus* are 1.21 (1.13-1.29) and 1.56 (1.42-1.70), respectively, whereas *D. c. radiolatus* are 1.17 (1.04-1.38) and 1.58 (1.46-1.70), respectively. These measurements from the 2 different hosts were not found to be statistically different (T-test) and all other parasite characteristics were similar, therefore,

measurements were pooled to produce a more accurate picture of the averages and ranges of *I. concentrica.*

DISCUSSION

Although the sporulated oocysts of *Isospora magna* and *I. concentrica* are similar in general shape and there is some overlap in size, there are considerable differences in sporocyst and sporozoite structure. *I. magna* sporocysts are ovoid with a dome-like Stieda body and an inconspicuous substiedal body while *I. concentrica* has more ellipsoidal sporocysts with prominent, square-shaped Stieda bodies and bubble-shaped substiedal bodies.

The contrast between the sporozoites of the two species is even more noticeable. The sporozoites of *I. magna* are sausage-shaped with refractile bodies at each end compared to the vermiform shaped sporozoites of *I. concentrica* with a refractile body at one end and its distinctive concentric markings at the tapered end.

Dendrocolaptes certhia is 1 of 5 species in the genus. It frequently feeds on arthropod prey fleeing army ants (Oniki and Willis 1972). In northwestern South America, its range is disjunct with no known connection between the Amazonian and west Andean-north Colombian subspecies (Hilty and Brown 1986), although this may be an artifact of limited collecting in Amazonian Colombia. The Amazonian (east-Andean) form in Ecuador is recognized as a subspecies, *D. c. radiolatus,* different from the west-Andean form in Ecuador, *D. c. columbianus* (Peters 1951). The 2 subspecies differ in plumage and vocalization (Hilty and Brown 1986) as well as show substantial frequency differences in isozymes (Brumfield 1993). Although these differences between the 2 avian host subspecies suggest species-level ranking, there were no significant differences between *Isospora concentrica* oocysts isolated from these Andean-separated hosts.

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ACTA
PROTOZOOLOGICA

Trypanosoma microhylii sp. n. from an Indian Anuran Amphibia, *Microhyla ornata*

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Summary. A new species of trypanosome from an Indian microhylid frog, *Microhyla oranata* is described from India. The trypomastigote form is small and always displays a peculiar configuration in fixed blood smear. Nucleus and kinetoplast are closely located at a distance from the posterior tip of the parasite. The trypanosome is given a new species status, *Trypanosoma microhylii* (Kinetoplastida).

Key words. Anuran frog parasite, Haemoflagellate, Kinetoplastida, Microhylidae, monomorphic trypanosome, *Trypanosoma microhylii* sp. n.

INTRODUCTION

Substantial works on the blood parasites of different members of amphibia have been done in various parts of the world. Though the bulk of the observations were restricted to the amphibian haemogregarines, many trypanosome species have also been recorded from urodel and anuran members particularly from African toads and frogs, including *Trypanosoma mega, T. rotatorium, T. inopinatum,* etc. Besides these species, *T. grylli* (Nigrelli 1944), *T. pipientis* and *T. schmidti* (Diamond 1950, 1965), *T. boyli* (Lehman 1959) and *T. canadensis* (Woo 1969) are important. *Trypanosoma bufophlebotomi* from the western toad, *Bufo boreas* (Anderson and Ayala 1968), *T. pseudopodium* from *B. americanus* from Michigan (Werner and Walewski 1976), *T. andersoni* from *Hyla versicolor* (Reilly and Woo 1982) and *T. fallisi* from *B. americanus* from Canada (Martin and Desser 1990) are the recent additions to anuran trypanosomes. In India altogether 10 species of anuran trypanosomes are so far recorded from 13 anuran hosts of which nine trypanosome species are observed in West Bengal (Ray and Choudhury 1984, Das et al. 1993). In the present study 8 species of anuran amphibian (*Bufo melanostictus, Kaloula pulchra, Microhyla ornata, Rhacophorus* sp., *Rana cyanophlyctis, R. hexadactyla, R. limnocharis limnocharis* and *R. tigrina*) are examined and

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infection of trypanosome is found in 3 individuals of a total of 18 microhylid frog, *Microhyla ornata* and is described as a new species. Infection of haemogregarine is found in *Bufo melanostictus, Rana tigrina* and *R. hexadactyla*.

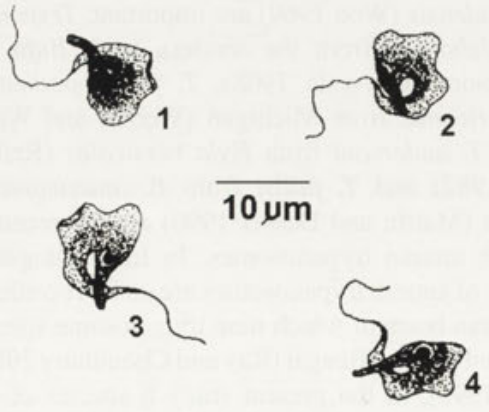
MATERIALS AND METHODS

Microhyla ornata (and other frogs and toads) were collected from different districts of West Bengal (India) mainly from Calcutta, Midnapore, and North and South 24-Parganas. Animals were kept either in large water vessels or in baskets having sufficient provision for moisture in accordance with their habit and habitat. They were supplied with ant eggs, earthworms, crickets and cockroaches. The animals were bled either by cutting the digit or by heart puncture. Blood smears were drawn on clean slides. Organ impression smears were also made. Air-dried smears were fixed in absolute methanol (acetone free) followed by Giemsa stain. Leishman stain was also used. Measurements were taken from Camera lucida drawings. Photographs were taken with the help of Leica camera.

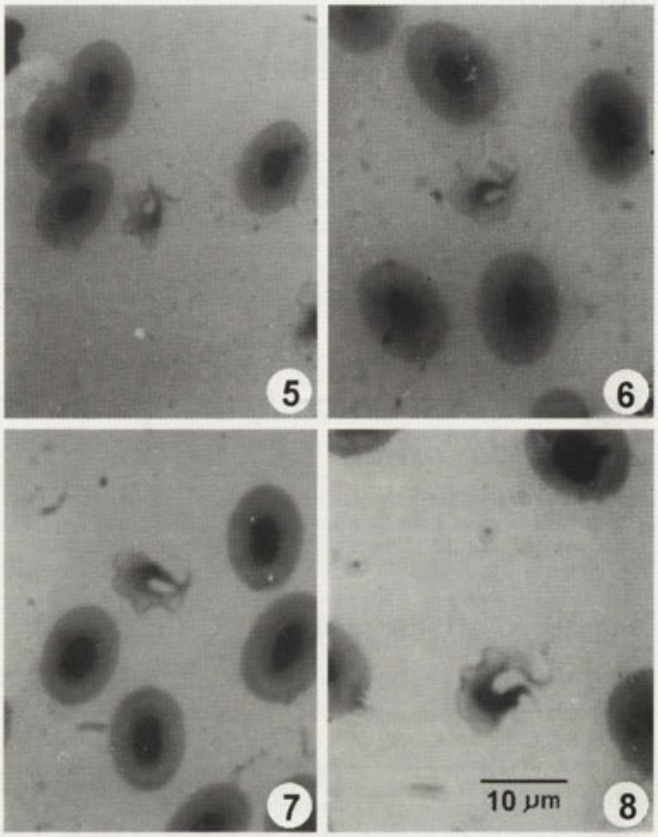
OBSERVATION

Trypanosoma microhylii sp. n.

The haemaflagellate as found in the heart blood smear (rare in circulation) is monomorphic (Figs. 1-8). Live trypanosomes are seen to move with jerk. The characteristic wriggling movement of the trypanosome is not observed. The cell body of the parasite is attenuated anteriorly and is blunt in the posterior end. Interestingly, in both unstained and stained smears, this trypomastigote form always displays a conspicuous configuration, the posterior part of the parasite arching over the anterior part. The posterior end is narrowly processed ending bluntly at its tip and a distinct post-kinetoplast region is present. The kinetoplast is a discoid body, stains intensively in Giemsa, situated towards the posterior end of the body and is adjacent to the cell membrane. The flagellum, originating from the kinetoplast, forms a conspicuous undulating membrane touching the cell body continuously (may form 4-6 apparent "waves" over its length) and leaves the latter as a distinct long free flagellum.



Figs. 1-4. Camera lucida drawings of *Trypanosoma microhylii* sp. n. from *Microhyla ornata*. Note the body contour and relative positions of the kinetoplast and the nucleus



Figs. 5-8. Photomicrographs of *Trypanosoma microhylii* sp. n. Note the contour of the haemoflagellate and the undulating membrane

Nucleus of the trypanosome is round, situated just anterior to the kinetoplast and adjacent to the cell membrane. It also stains intensively in Giemsa. The cytoplasm is granular. Because of the diffused staining response of the cell body and the undulating membrane, it is often very difficult to distinguish them separately. It poses a problem to get the actual measurement of the width of the haemoflagellate. However, a certain region of the cell cytoplasm stains deep red and the remaining apparent undulating membrane stains faintly and uniformly. Measurement of the trypanosome is given in Table 1.

Divisional stage of this trypanosome is not observed in either the blood film or in the organ smears. Heart

Table 1

Mensural data (in µm) of *Trypanosoma microhylii* sp. n. from anuran host, *Microhyla ornata*

Particulars	Range	Mean ±S.D.	S.E.	C.V
Total length of the body (including free flagellum)	33.0- 39.0	36.8 ±1.75	0.553	0.047
Length of the cell body	19.0 - 25.0	22.8 ±2.0	0.634	0.086
Length of the free flagellum	13.0 - 16.0	14.0 ±0.96	0.306	0.069
Breadth of the body (including undulating membrane at the minimum point)	4.0 - 6.0	5.1±0.58	0.184	0.114
Nucleus (diameter)	-	0.5 x 1.0	-	-
Post-kinetoplast distance	4.0 - 7.0	5.1 ±0.86	0.272	0.168
Distance between kinetoplast and nucleus	0.5 - 1.5	1.0 ±0.38	0.122	0.387

blood (diluted with citrate saline 1:1) of one infected *Microhyla ornata* is inoculated intraperitonealy to 2 clean *Rana limnocharis limnocharis*. However, no trypanosome is detected in the blood of those frogs till fifteen days after inoculation.

Prevalence: 3 out of 18 (16.5%) individuals of *Microhyla ornata* were positive for trypanosome.

Type material: holotype in one slide and paratypes in 5 slides are deposited in the Protozoology Laboratory, Department of Zoology, Calcutta University.

Type host and locality: *Microhyla ornata*, Calcutta, India. 10.X.1972. Coll. A.K. Chandra.

DISCUSSION

The anuran host, *Microhyla ornata* collected from Nova Goa, India, was previously known to harbour *Trypanosoma chattoni*, Mathis and Leger (Ray 1980, Ray and Choudhury 1983). *Trypanosoma taprobanica* Ray and Choudhury was described from another member of the family Microhylidae, *Kaloula pulchra taprobanica*, collected from Santaldi, West Bengal, India (Ray and Choudhury 1983, 1984). The present trypanosome from *Microhyla ornata* is completely different from *T. chattoni* which is almost a globular trypanosome, the generic status of which is under scrutiny (Diamond 1965). *Trypanosoma taprobanica* from *Kaloula pulchra taprobanica* is apparently similar to the trypanosome described at present. However, the haemoflagellate under discussion is much larger than that of *T. taprobanica* in all the mensural aspects (Table 2). The other two species of anuran trypanosomes described from India, *T. malabarica* from *Rana malabarica* and *T. systoma* from *Uperodon systoma*, also differ from the present species both morphologically as well as mensurally. Other 6 species of anuran trypanosomes recorded from India, viz., *T. rotatorium, T. inopinatum, T. loricatum, T. karyozeukton, T. mega* and *T. ranarum* differ from the present species in detail of measurement and morphology (cf. Ray and Choudhury 1983, Das et al. 1993). The present species also differs from *T. bufophlebotomi, T. pseudopodium*, and *T. fallisi* described from American toad in the shape and measurement of the parasite. This species is much smaller than the trypanosomes mentioned.

Considering the above comparison the trypanosome from *Microhyla ornata* is assigned a new species status

Table 2

Comparison of the mensural data of *T. taprobanica* (Ray and Choudhury 1983) described from *K. p. taprobanica* and *T. microphylii* sp. n. from *M. ornata*

Mean (in µm)	*T. taprobanica* from *K.p. taprobanica* (Ray and Choudhury, 1983)	Present *Trypanosoma* from *M. ornata*
Total length (including free flagellum)	25.94	36.8
Length of the cell body (PA)	16.64	22.8
Free flagellum (FF)	8.30	14.0
Breadth of the cell body (BW)	1.84 (excluding undulating membrane)	5.1 (including undulating membrane at the minimum point)
Nucleus diameter	1.0 x 0.59	1.0 x 0.5
Post kinetoplast distance (PN)	3.20	5.1
Kinetoplast and nucleus (KN)	1.30	1.5
FF/PA	0.49	0.657

as *Trypanosoma microhylii* after the generic name of the host.

Acknowledgement. The authors gratefully acknowledge Prof. A. Choudhury, Department of Marine Science, Calcutta University for his advice, during the work. The authors also thank Head of the Department of Zoology, Calcutta University for providing necessary laboratory facilities.

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ACTA
PROTOZOOLOGICA

A New Myxosporean, *Bipteria indica* sp. n. (Myxospora: Sinuolineidae) from the Gall Bladder of the Striped Mullet, *Mugil cephalus* Linnaeus

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Summary. A new myxosporean, *Bipteria indica* sp. n., a parasite of the gall bladder of the striped mullet, *Mugil cephalus* under brackish water conditions is described from Visakhapatnam, Andhra Pradesh, India is described. *B. indica* is the first record of and only species in the genus from the gall bladder in brackish water fish.

Key words. Myxosporean, *Bipteria indica* sp. n., *Mugil cephalus*, gall bladder.

INTRODUCTION

Genus *Bipteria* (Kovaleva et al. 1983) is characterised by the presence of inversely pyramidal spores with sinuous sutural line; anterior end of each shell valve extending into wing like projections in valvular view with remnants of valvogenic nuclei; spherical polar capsule; single sporoplasm and trophozoites di or polysporous; coelozoic in the urinary system of marine fishes (Lom and Noble 1984). Kovaleva et al. (1983) described 3 species namely, *B. admiranda* from the sparid, *Pagellus acarne, B. minima* and *B. magna* from the macrourid, *Coryphaenoidis pectoralis*, all from the urinary bladder of fishes of Atlantic and Pacific Oceans.

During the course of an investigation on the parasites of mullets in the backwater regions of Visakhapatnam, Andhra Pradesh, India, a new myxosporean was encountered in the gall bladder of the striped mullet, *Mugil cephalus* which is described in this paper.

MATERIALS AND METHODS

Specimens of the striped mullet, *Mugil cephalus* were collected from the backwater regions of Visakhapatnam harbour and Gosthani estuary located 30 km north of Visakhapatnam, Andhra Pradesh, India. Immediately after collection, the fish were autopsied and their gall bladder was carefully isolated. Smears prepared from the bladder contents were then either air dried, fixed in methyl alcohol and stained with Giemsa, with or without hydrolysis in 1 N HCl at 60°C or wet fixed in Schaudinn's or Carnoy's fluid and stained with haematoxylin or with Lugol's iodine for the detection of iodinophilous vacuoles and negatively with India ink for spore appendages. Hydrogen peroxide or saturated aqueous urea were used to induce extrusion of polar

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filaments. The diagrams were drawn with the aid of camera lucida and all measurements are in µm and were taken with an ocular micrometer

RESULTS

Altogether 1260 of 2344 (53.7%) individuals of *Mugil cephalus* were found infected with the parasite and no apparent pathogenicity of the parasite was observed.

Bipteria indica sp. n., (Figs. 1-4)

Description: development coelozoic, plasmodial stages 2.3-3.8 x 3.0-4.5 with 1 or 2 nuclei, adherent to the free surface of epithelial cells of gall bladder. Motile plasmodia, with 4-12 nuclei, floating freely in the bile. Pansporoplasts, broadly oval or irregular, disporous, 7.5-12.0 x 6.0-10.0. Sporoblasts, arranged diagonally in opposite direction (Fig.1). Fresh spores, inversely pyramidal. Anterior end broad with rounded corners. Posterior end, narrow. Spore valves thin, smooth, refractile and appear glossy in fresh condition. Sutural line curved at posterior end. Two delicate wing-like appendices arise from the anterior region of spore and extend up to posterior end almost enclosing the spore body (Fig. 2). Polar capsules spherical, widely spaced on either side of sutural line. Intercapsular appendage deeply stained. Polar filament forms 5-6 coils while inside capsule sporoplasm binucleate, occupies entire extracapsular region (Fig. 3). Iodinophilous vacuole absent. Polar filaments thin and uniform when fully extended (Fig. 4)

Spores: (n=50), 5.6-6.8 (5.8) in length, 5.4-6.8 (5.7) in width, 4.8-5.2 (5.0) thick. Appendage, 1.5-2.8 (1.8). Polar capsule, 1.8-2.6 (2.0), polar filament, 16.0-20.0 (18.0).

Type host: *Mugil cephalus* Linnaeus

Type locality: Visakhapatnam, Andhra Pradesh, India

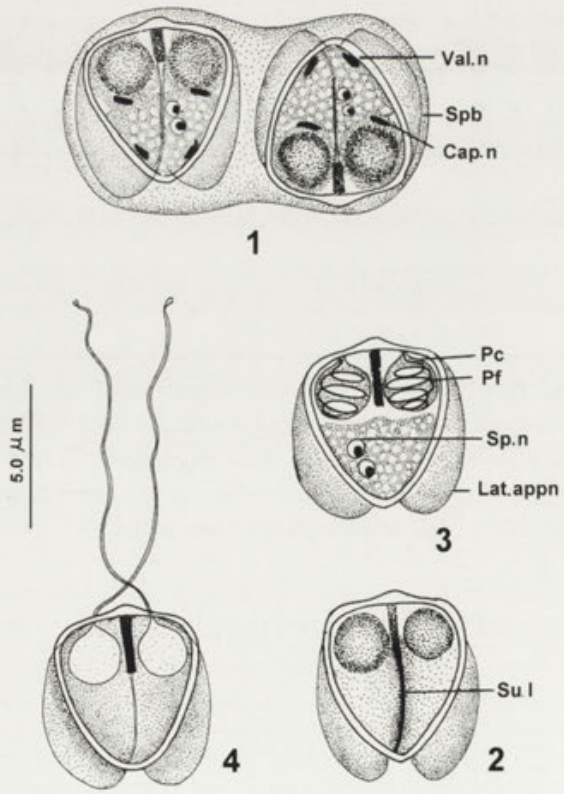
Prevalence: 1260 out of 2344 (53.7%) striped mullets infected.

Site of infection: gall bladder.

Types: stained slides and material fixed in Schaudinn's fluid in authors' collection at the Department of Zoology, Andhra University, Visakhapatnam.

Syntypes: stained slides with Zoological Survey of India Museum, Calcutta.

Etymology: the specific name 'indica' is after the Indian subcontinent.



Figs. 1-4. Stages of *Bipteria indica* sp. n. 1 - pansporoblast, 2 - fresh spore, 3 - a giemsa stained spore, 4 - a spore with extruded polar filaments. Abbreviations: Cap. n - capsulogenic nuclei, Lat. appn - lateral appendage, Pc - polar capsule, Pf - polar filament, Spb - sporoblast, Sp. n - sporoplasmic nuclei, Su. l - sutural line, Val. n - valvogenic nuclei

DISCUSSION

So far, only 3 species, namely *Bipteria admiranda, B. minima* and *B. magna* have been described in the genus *Bipteria* (Kovaleva et al. 1983). In comparison, the spores of *Bipteria indica* sp. n. are much smaller (5.8 x 5.7 x 5.0) than those of *B. admiranda* (10.64-13.96 x 10.64-11,97 x 13.3), *B. minima* (10.13-13.36 x 10.264 - 13.3 x 16.6 - 17.2) or of *B. magna* (15.96-18.62 x 13.3-15.96 x 21.3-27.0). Further, the three earlier species were parasites in the urinary bladder of deep water fishes, the present form was encountered in the gall bladder of a brackish water fish, *M. cephalus*. Moreover, *B. indica* sp. n. is charecterised by having pyramidal spores with lateral appendices enclosing spore body, a posteriorly curved sutural line, deeply stained intercapsular appendix and a unique glossy appearance in fresh condition. In view of these differences in spore measurements, characteristic morphology and based on its occurrence in an altogether new host, a mullet

(*M. cephalus*) in brackish water, in a totally new locality, the present species is considered new to science for which the name *Bipteria indica* sp. n. is proposed.

Acknowledgements. We are thankful to the Department of Environment, New Delhi for grant-in-aid and to the Head of the Department of Zoology, Andhra University for laboratory facilities. We are grateful to Dr. A.V. Raman, Division of Marine Biology, Department of Zoology for help during the study.

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ACTA
PROTOZOOLOGICA

Biosystematics of Two New Species of *Stenoductus* (Apicomplexa: Sporozoea)

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Summary. The morphology and life history of 2 new species of cephaline gregarines infecting two millipede species are described in this paper. These are, *Stenoductus cannanorensis* sp. n. from *Janardananeptus cannanorensis* and *S. demangei* sp. n. from *Harpurostreptus prasadani*.

Key words. *Stenoductus cannanorensis* sp. n., *S. demangei* sp. n., gregarines, millipedes, *Janardananeptus cannanorensis*, *Harpurostreptus prasadani*.

INTRODUCTION

The genus *Stenoductus* was established by Ramachandran (1976), with *S. penneri* from *Floridobolus penneri* as its type. Till date 10 species were added to the genus from Indian millipedes (Janardanan and Ramachandran 1979, 1981a, b, 1982a, b, 1983a, b; Janardanan 1987; Prema and Janardanan 1991).

During an explorative study on the cephaline gregarines infecting millipedes in Kerala, the authors came across 2 new species of *Stenoductus* in the guts of the millipedes, *Janardananeptus cannanorensis* and *Harpurostreptus prasadani*. These species are herein described and their systematic position discussed.

MATERIALS AND METHODS

The millipedes, *Janardananeptus cannanorensis* Demange collected from Ezhimala in Cannanore district and Mothanga in Wayanad district of Kerala and *Harpurostreptus prasadani* Demange from Calicut University Campus, in Malappuram district of Kerala, were brought alive to the laboratory and immediately examined for their gregarines, or were maintained alive and studied at convenience. Sporadins and trophozoites recovered from the midguts and gametocysts from the hindguts/faecal matter were studied following Prema and Janardanan (1987). Photographs of development stages of gregarines were taken with the help of a Wild MPS 12 micro camera fitted to a Leitz Diaplan phase-contrast microscope. Illustrations were made with the help of camera lucida; descriptions are based on the measurements of a minimum of 20 specimens. Measurements are in micrometers (µm); the range is followed by the mean value (in parentheses).

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Abbreviations used in this paper are: DW - deutomerite width, PL - protomerite length, PW - protomerite width, TL - total length.

RESULTS

Stenoductus cannanorensis sp. n.

Host: *Janardananeptus cannanorensis* Demange

Site: intestine.

Locality: Ezhimala in Cannanore district and Mothanga in Wayanad district, Kerala (India).

Date of collection: June to December 1987, 1988 and 1989.

Holotype: to be deposited in the parasite collections, Parasitology Laboratory, Department of Zoology, University of Calicut, Kerala (India).

Description

Sporadins (Figs. 1A, 2D): solitary, milky-white, almost cylindrical with a round caudad. Protomerite dome-like, wider than long; apex papillate, papilla 7.7-9.6 in diameter; apical pore present, closed by a fine layer of epicyte; protomerite epicyte uniformly thick, longitudinally striated, striations continuous with those on deutomerite; endocyte of protomerite granular; septum circular, slightly convex toward deutomerite, 3.8 thick; constriction at septum conspicuous. Deutomerite elongate, almost cylindrical with a round caudad. Deutomerite epicyte uniformly thick, striated; endocyte granular. Nucleus fusiform, not visible in fresh sporadins, variable in position; its location indicated by a relatively clear region; nucleolus round to ovoid. Nucleus in sporadin of 889.3 by 100.1 measured 84.7 by 26.9.

Measurement of sporadins in µm (with mean in parentheses) are noted below:

DW = 57.7-100.1 (83.9); PL = 23.1-34.7 (27.3)

PW = 38.5-80.8 (63.7); TL = 512-889.3 (758.9)

Ratios: PL:TL = 1:27.8; PW:DW = 1:1.3

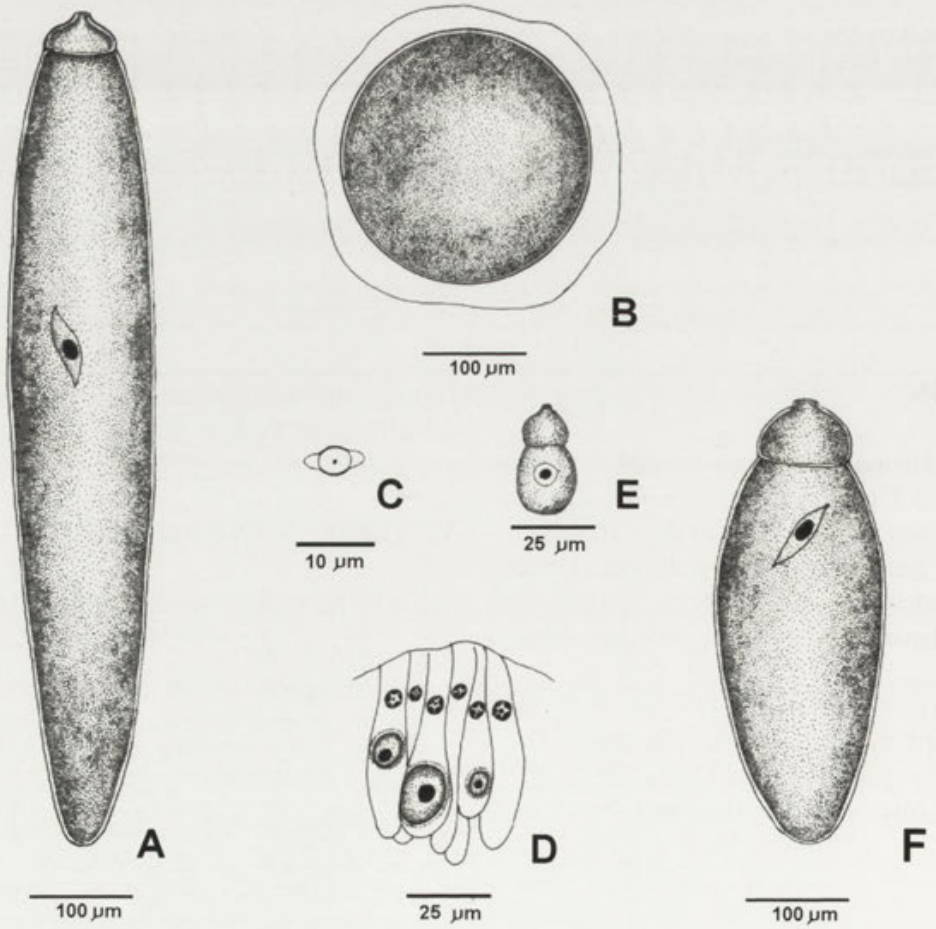

Fig. 1 A-F. *Stenoductus cannanorensis* sp. n. A - sporadin;B - gametocyst; C - spore; D - midgut epithelial cells showing intracellular aseptate trophozoites; E, F - lumen trophozoites

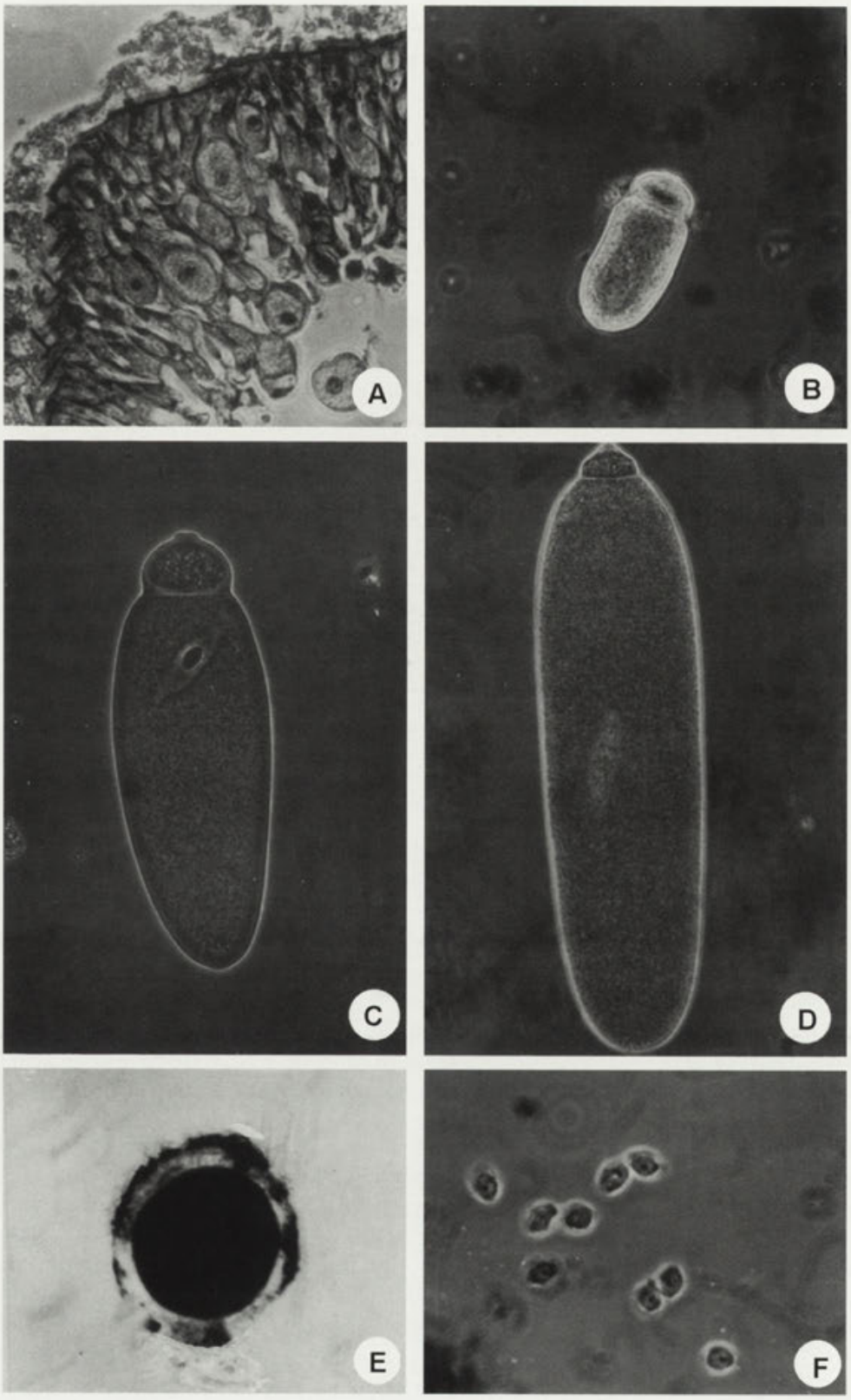

Fig. 2 A-F. *Stenoductus cannanorenis* sp. n. A - midgut epithelial cells showing intracellular aseptate trophozoites (x 530); B - lumen trophozoite (x 530) ; C - lumen trophozoite (x 160); D - sporadin (x 160) ; E - gametocyst (x 115); F- spores (x 890)

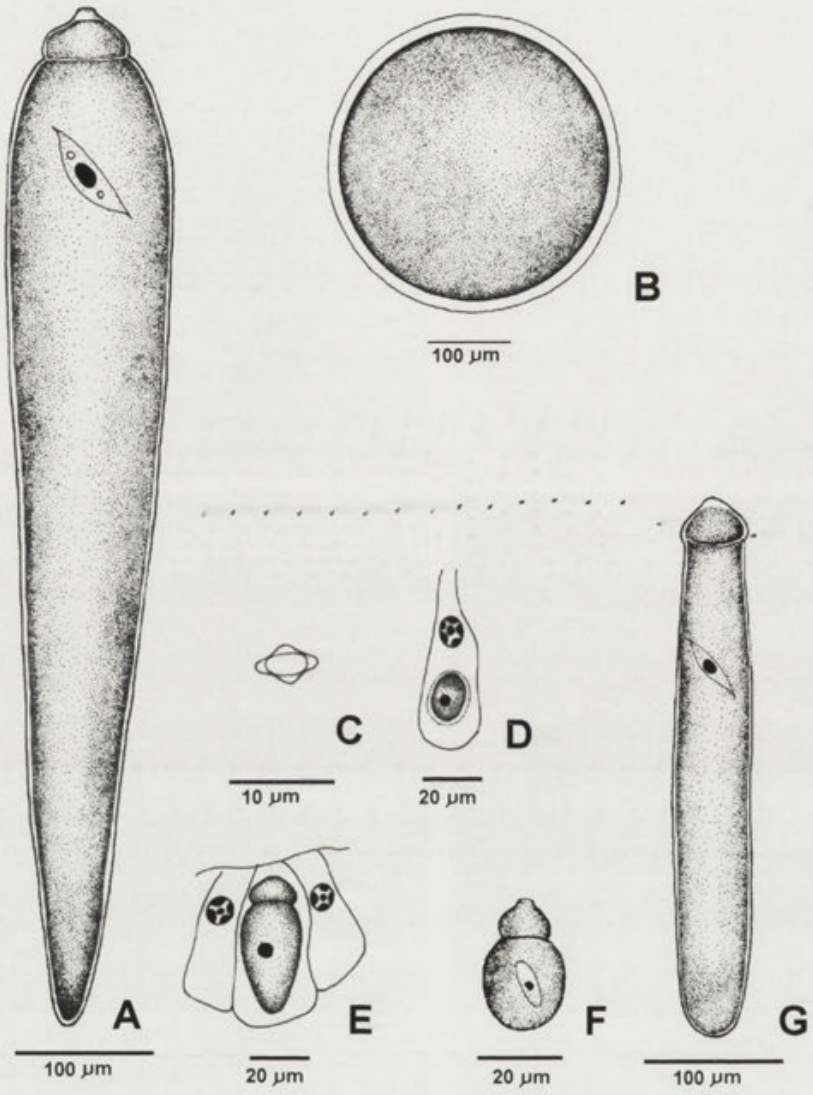

Fig. 3 A-G. *Stenoductus demangei* sp. n. A - sporadin; B - gametocyst; C - spore; D - midgut epithelial cell showing an intracellular aseptate trophozoite; E - midgut epithelial cells showing an intracellular septate trophozoite; F, G - lumen trophozoites

Gametocysts (Figs. 1B, 2E): spherical, milky-white, opaque; cyst wall two layered; endocyst regular, 3.8 thick; epicyst irregular, hyaline, 30.8 to 46.2 thick; line of association clear in newly formed cysts. Fresh gametocysts measured 227.1-292.6 (251). Sporoduct naked, cord-like, measured 1555.4-1732.5 long; spores embedded along the sporoduct.

Spores (Figs. 1C, 2F): ovoid, with a hyaline epispore, forming hemispherical polar projections; endospore thick, dark brown. Fresh spores with epispore measured 7.5 by 3.4.

Sporozoites: vermiform, octozoic.

Development: gametocysts kept in moist chamber developed spores in 4 days. Spores were extruded in a naked, cord-like sporoduct which came out through a rupture in the cyst wall. The sporoduct disintegrated slowly by absorbing moisture and set the spores free. The liberation of sporozoites was induced by placing fresh spores in the millipede's midgut fluid which activated the sporozoits in 4 min. The sporozoites came out through an opening at one pole of the spore after 5 min. Millipedes seem to pick up infection by ingesting viable spores. The sporozoites developed into trophozoites (Figs. 1E, F; 2B, C) in the midgut epithelial cells. The intracellular

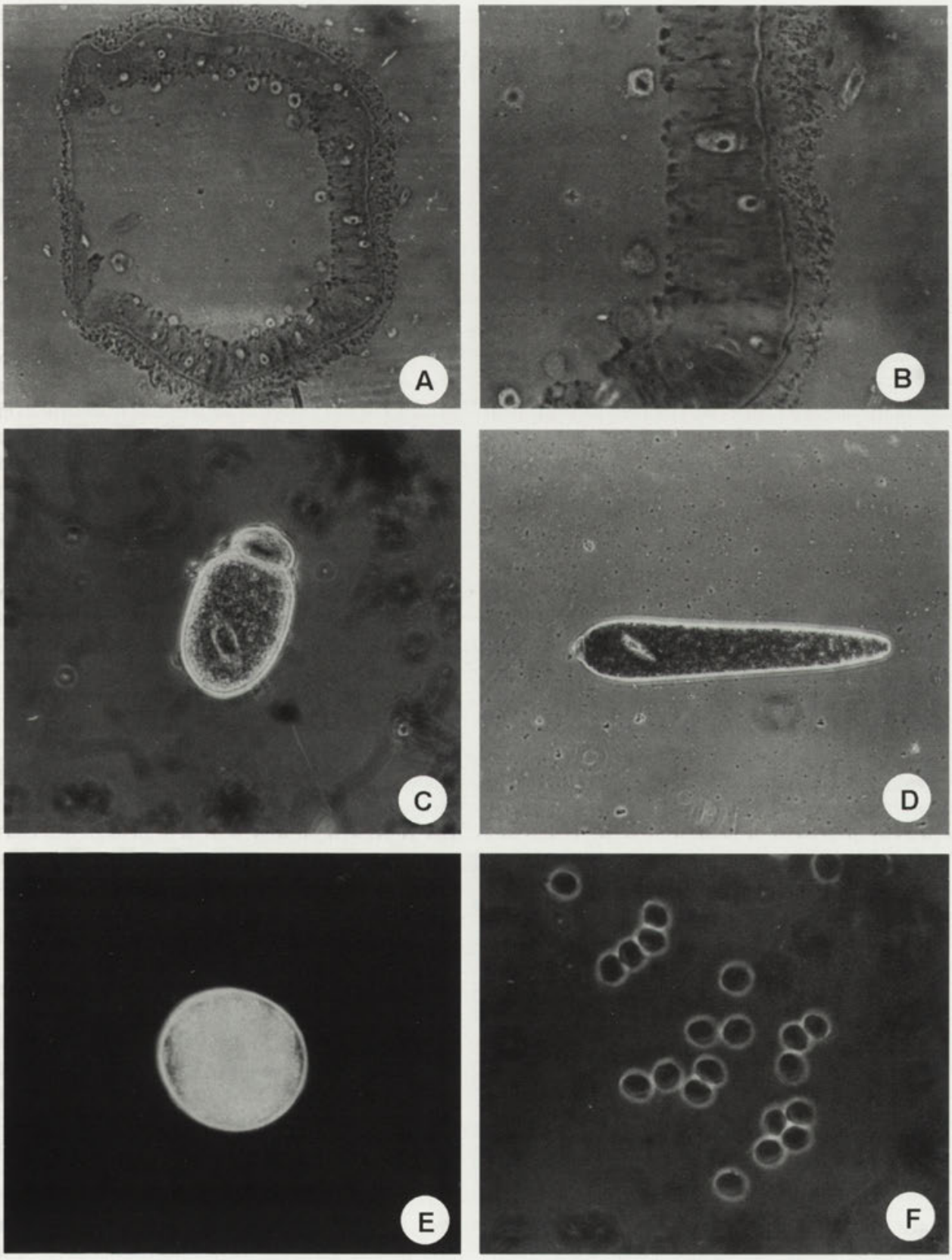

Fig. 4 A-F. *Stenoductus demangei* sp.n. A- cross section of midgut showing intracellular trophozoites (x 85), B- cross section of midgut showing intracellular trophozoites (x 200), C- lumen trophozoite (x 530), D- sporadin (x 85), E- gametocyst (x 115), F- spores (x 890)

trophozoites (Figs. 1D, 2A) observed were uninucleate, aseptate, ovoid bodies, measuring 6.6-19.8 by 4.9-13.2.

***Stenoductus demangei* sp. n.**

Host: *Harpurostreptus prasadani* Demange

Site: intestine.

Locality: Calicut University Campus in Malappuram district, Kerala (India).

Date of collection: June to September 1988, 1989 and 1990.

Holotype: to be deposited in the parasite collections, Parasitology Laboratory, Department of Zoology, University of Calicut, Kerala (India).

Description

Sporadins (Figs. 3A, 4D): solitary, milky-white, broader at the anterior region and gradually tapering to a narrow round caudad. Protomerite dome-shaped, wider than long; apex papillate, papilla 7.7-9.6 in diameter; apical pore present, closed by a thin layer of epicyte; protomerite epicyte thin, uneven, longitudinally striated, striations continuous with those on deutomerite; endocyte of protomerite granular with a small granule-free area at the anterior region; septum circular, slightly convex toward deutomerite, 3.8 thick; constriction at septum conspicuous. Deutomerite elongate, a little broader at the anterior, gradually tapering to a narrow round caudad; epicyte hyaline uniformly thick, longitudinally striated; endocyte granular. Nucleus fusiform, feebly visible in fresh sporadins; its location indicated by relatively clear endoplasmic region, position of nucleus variable; nucleolus round to ovoid, deep staining with hematoxylin. Nucleus in a sporadin of 939.4 by 134.8 measured 107.8 by 30.8.

Measurement of sporadins in µm (with mean in parentheses) are noted below:

DW = 69.3-157.9 (107); PL = 28.9-38.5 (32.2)

PW = 46.2-65.5 (52.6); TL = 562.1-939.4 (811.8)

Ratios: PL:TL = 1:25.2; PW:DW = 1:20

Gametocysts (Figs. 3B, 4E): spherical or ovoid, opaque, milky-white, with a single hyaline cyst wall, 11.6-30.8 thick; line of association clearly visible in newly formed cysts. Spherical gametocysts measured 130.9-207.9 (176.3); ovoid cysts measured 184.8-204 by 161.7-169.4. Sporoduct naked, cord-like; spores embedded along the whole length of sporoduct.

Spores (Figs. 3C, 4F): ovoid, with a hyaline epispore forming hemispherical polar projections and a conical equatorial ridge; endospore thick, dark brown; 2-8 dark bodies present inside the spores. Fresh spores with epispore measured 6.8 by 4.5-4.9 (6.8 by 4.7).

Sporozoites: vermiform, octozoic.

Development: gametocysts maintained in moist chamber developed spores in 11 days. The sporoduct came out through a rupture in the cyst wall; the naked cord-like sporoduct disintegrated slowly by absorbing moisture and sets the spores free. The liberation of sporozoites was induced by placing fresh spores in the host's midgut fluid, which activated the sporozoites in 4 min. The activated sporozoites came out through a pole of the spore in 5 min. The hosts seem to pick up infection by ingesting viable spores.

The sporozoites penetrate the midgut epithelial cells and develop into trophozoites. The smallest observed intracellular trophozoite (Fig. 3D) was aseptate, ovoid, 13.2 by 8.3, stainable with hematoxylin, but for a halo of unstained host cell region around; nucleus centrally located. Largest observed intracellular trophozoite (Figs. 3E; 4A, B) was septate, measured 46.2 by 19.8 with a 9.9 long protomerite and 36.3 long deutomerite. The host cell enlarged progressively with the growth of the trophozoite. Trophozoites of same size and larger and smaller ones were observed in the midgut lumen. The smallest observed lumen trophozoite (Figs. 3F, 4C) measured 23.1 by 15.4; its protomerite was hemispherical with a small apical papilla, and deutomerite spherical with a centrally placed round nucleus.

DISCUSSION

The present gregarines from *J. cannanorensis* Demange and *H. prasadani* Demange have solitary sporadins without epimerites and their gametocysts extrude ovoid spores through naked, cord-like sporoducts. These characters amply justify the assignment of the gregarines to the genus *Stenoductus* Ramachandran, 1976 of the family Monoductidae Ray and Chakravarty, 1933 (Levine, 1988).

Stenoductus cannanorensis sp. n.is comparable to *S. ktenostrepti* Janardanan, 1987 from *Ktenostreptus calcaratus* in sporont morphology. But a comparison of characters clearly shows that the present form is significantly different in measurements and ratios and in having: (1) almost cylindrical deutomerite, (2) fusiform nucleus with round to ovoid nucleolus, (3) spherical gametocysts with uneven, hyaline epicysts and (4) ovoid spores without equatorial ridge. Besides, this is the first report of a cephaline gregarine from the present host and locality. The gregarine is therefore, considered a new species and is reported here as *Stenoductus cannanorensis* sp. n., after its host.

The gregarine *Stenoductus demangei* sp. n. from *Harpurostreptus prasadani* Demange resembles most to *S.. carlogoni* Janardanan and Ramachandran, 1979, infecting *Carlogonus palmatus* and to *S. organognathi* Janardanan and Ramachandran, 1982 infecting *Organognathus janardhanani* and *Fageostreptus hyatti*. A comparative study of characters clearly shows that the gregarine is significantly different from *S. carlogoni* and *S. organognathi* in measurements and ratios, and in having (1) protomerite with a granule-free region at the anterior end, (2) deutomerite with broad anterior region which is gradually tapering to a narrow round caudad, (3) fusiform nucleus with round to ovoid nucleolus and two extra nucleoli bodies and (4) trophozoites without epimerites. Further, this is the first record of a gregarine from the present host and, therefore, it is proposed to name the species *Stenoductus demangei* sp. n. in honour of Professor J.M. Demange of the Natural History Museum, Paris, who identified the host.

Acknowledgements. The authors express their sincere thanks to Prof. J.M. Demange, Natural History Museum, Paris for identification of the host specimens. One of the authors (P.K.P.) is grateful to C.S.I.R., New Delhi for financial assistance.

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Short communication

High Molecular Weight Proteins Associated with the Basal Body in African Trypanosomes

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Summary. Immunoblots of protein preparations from *Trypanosoma brucei* were probed with monoclonal antibodies directed against basal-body associated proteins in *Tetrahymena* called tetrins. An antibody recognizing tetrin 4 in *Tetrahymena*. mAb11C7, blotted a double band at about 300 kDa in total cell protein preparations and in detergent-extracted cytoskeletons of *T. brucei*. Immunofluorescence preparations using triple labeling with antitubulin, mAb11C7, and DAPI showed that mAb11C7 stains three small dots close to the kinetoplast very brightly. Immunogold preparations of both whole mount and thin sections of cytoskeletons examined with the electron microscope showed that the mAb11C7-positive material is located in the dense cloud of material surrounding the flagellar basal body. The same results were obtained with other African trypanosomes, but similar high molecular weight (HMW) proteins were not found by these procedures in South American forms. Evidence is presented indicating that the tetrin-related proteins in *T. brucei* are heavily phosphorylated.

Key words. Monoclonal antibodies, Western blots, immunofluorescence, immunoprecipitation.

INTRODUCTION

Tetrins are filament-forming proteins of molecular weight 79-89 kDa first reported in association with basal bodies in the oral apparatus of *Tetrahymena* (Honts and Williams 1990, Dress et al. 1992). They were subsequently found to be present also in the oral apparatus of *Paramecium* (Keryer et al. 1990). *Tetrahymena* tetrins have been well characterized (Honts and Williams 1990) and a number of tetrin-specific monoclonal antibodies has been obtained Dress et al. 1992). One of us (N. E. W.) noticed recently that detectable amounts of tetrin appear transiently around the somatic basal bodies during cell division in *Tetrahymena* (unpublished), and anti-tetrin antibodies stain the somatic basal bodies of some ciliates at all stages in the cell cycle, e.g. apostomes (Williams 1986).

Finding tetrins associated with basal bodies in a number of ciliates raises questions as to how widespread these proteins are and what their functional significance might be. We have stained a number of vertebrate cells with anti-tetrin antibodies in an attempt to see if tetrins might be a universal basal body and/or centrosomal component, so far without success. It has been reported, however, that tetrin 1 is recognized by mAbCG3, which recognizes vertebrate tropomyosin, and that tetrin 2 is recognized by the Pruss antiintermediate filament antibody (Honts and Williams 1990).

The extent of distribution of tetrin or tetrin-related proteins remains an open question. It is possible that

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they may be more widespread than we know at present, and consequently they may be of more general significance than is now recognized. At least one universally acknowledged centrosomal protein, centrin or caltractin, was first discovered in studies of the basal body apparatus in flagellated protozoa (Lee and Huang 1993, Errabolu et al. 1994).

In the present study, we have tested a number of anti-tetrin monoclonal antibodies for recognition by *Trypanosoma* cells in an attempt to see if tetrins might be present in these medically important flagellates. We report here the recognition of a 330-kDa protein in African trypanosomes by anti-tetrin mAb11C7, and find that it is intimately associated with the flagellar basal body.

MATERIALS AND METHODS

Parasites

Trypanosoma brucei procyclic cultures were grown in SDM-79 medium as described (Brun and Schoenenberger 1979) and were harvested during exponential growth (i.e. five times 10^6 cells/ml. *T. congolense* bloodstream forms were cultivated in RPMI/IMDM medium (Sigma) according to Hemphill et al. (1994).

T. congolense epimastigotes were a kind gift of Dr Carole A. Ross (Centre for Tropical Veterinary Medicine, University of Edinburgh). *T. brucei* bloodstream forms, *T. cruzi* amastigotes, trypomastigotes and epimastigotes were provided by Dr Simon L. Croft (London School of Hygiene and Tropical Medicine). Promastigotes of different *Leishmania* species (*L. major, L. tropica, L. infantum, L. mexicana* and *L. braziliensis*) were obtained from Dr Robert Harris (Karolinska Institut, Stockholm).

Preparation of extracts for SDS-PAGE

Whole cell lysates of different *Trypanosoma* and *Leishmania* species were prepared by centrifugation of cultures which were in exponential growth phase at 1,000 g for 10 min at 4°C. The parasites were washed twice in cold PBS (50 mM Na-phosphate, 150 mM NaCl, pH 7.2), and after the final centrifugation pellets were solubilized in 6 M guanidinium chloride. Protein concentrations were measured (Bradford 1976), and equal amounts of parasite material was analyzed by SDS-PAGE (Laemmli 1970).

Subcellular fractionation of *T. brucei* procyclics was performed at 4°C as previously described (Hemphill et al. 1991, 1992) Briefly, cells were washed in PBS and were incubated for 5 min on ice in MME-buffer (100 mM morpholino propane sulfonic acid (MOPS), 50 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, pH 6.9) containing the following protease inhibitors: leupeptin, chymostatin, and pepstatin at 5 µg/ml each, and PMSF at 0.2 mM. To half of the cells, Triton X-100 was added to 0.2% and the parasites were extracted for 5 min on ice. Whole cells and cytoskeletons were then collected by centrifugation at 5,000 g at 4°C. Triton-soluble extracts were transferred to fresh tubes, and the pellets (cytoskeletal fraction and whole cells) were resuspended in the same volume of MME-Triton-X-100. All three fractions (whole cells, triton-soluble- and cytoskeletal fraction) were then extracted and precipitated with methanol/chloroform (Wessel and Fluegge 1984). The final pellets were solubilized in SDS-PAGE-sample buffer, and fractions corresponding to the same amount of cells were analyzed by SDS-Page.

Tetrahymena pyriformis cortical residues were prepared by extracting the cells in Triton X-100 at high ionic strength as described by Williams et al. (1990).

Antibodies and immunoblotting

The ten monoclonal antibodies directed against tetrins from *Tetrahymena* (4G9, 2D10, 8C10, 9E9, 4B3, 1H7, 21A5, 2D3, 12F3 and 11C7) are described in Dress et al. (1992).

The polyclonal rabbit anti-tubulin antibody was a kind gift from Prof. Thomas Kreis, University of Geneva (Hemphill et al. 1992).

Protein extracts of *Tetrahymena*, of erythrocyte ghosts, *Trypanosoma* and *Leishmania* were separated by SDS-PAGE and transferred to nitrocellulose filters according to Towbin et al. (1979). Blocking of unspecific binding sites was carried out overnight at 4°C in PBS containing 3% BSA and 0.05% Tween-20. The monoclonal antibody culture supernatants were diluted 1:5 in PBS/0.3%BSA and were applied overnight at 4°C. Filters were then washed three times in PBS/BSA, and the bound antibodies were visualized using alkaline phosphatase-conjugated anti-mouse immunoglobulin anybodies (Cappel).

Triple labeling immunofluorescence

Parasites and cytoskeletons were mounted on glass coverslips as described in Mueller et al. (1992). The coverslips were subsequently incubated for 1 h in blocking buffer (PBS/0.5%BSA/ 50 mM lysine). Hybridoma culture supernatants of cells producing 11C7 monoclonal antibodies were diluted 1:1 in blocking buffer and were applied for 60 min at 24°C in a moist chamber. The second antibody was a goat anti-mouse-FITC (Cappel) and was used at a dilution of 1:100 in blocking solution. Thereafter staining of the microtubular skeleton was carried out with a polyclonal rabbit anti-tubulin antiserum at a dilution of 1:100 in blocking solution (Hemphill et al. 1992), and as a fourth layer a goat anti-rabbit conjugated to Texas red (Becton Dickinson Immunocytometry Systems) was used. After labeling, coverslips were extensively washed for 6 times 5 min in PBS, and were stained with DAPI (Sherwin et al. 1987). They were then mounted onto glass slides using a mixture of gelvatol/glycerol (Hemphill et al. 1992).

^{32}P labeling of *T. brucei* and immunoprecipitation using mAb11C7

Two times 10^8 parasites were harvested by centrifugation and were washed in ME-83 media (Seebeck and Kurath 1985). They were resuspended in 10 ml ME-83 containing 500 µCi disodium (^{32}P) orthophosphate (ICN). The cells were incubated for 3 h at 28°C, centrifuged and washed twice in PBS. They were then extracted in MME/ 0.2% Triton X100 as described above. The cytoskeletons were resuspended in 1 ml of 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% NP4O (TNE-buffer) containing the protease inhibitors chymostatin, leupeptin, pepstatin and PMSF, and were sonicated extensively This resulted in complete desintegration of the membrane skeleton, while residues of the flagellar skeleton remained undissolved. The fraction was spun for 15 min at 100,000 g at 4°C and the supernatant was collected.

In order to clear the supernatant (1 ml) of proteins which would bind unspecifically to Protein A-Sepharose, 100 µl of a 50% slurry of

Protein A-Sepharose (Sigma), prewashed three times in TNE plus proteinase inhibitors, was added and incubated for 4 h at 24°C with gentle agitation. The supernatant was cleared by centrifugation, then 1 ml of mAb11C7 culture supernatant was added and the mixture was incubated on a rotating table for 12 h at 4°C. This mixture was reacted for 2 h with an affinity purified rabbit anti mouse IgG (DAKO) at a dilution of 1:200, and finally 200 µl of prewashed Protein A-Sepharose was added and incubated for another 2 h at 24°C with gentle agitation. As control experiments supernatants were also incubated (i) with Protein A Sepharose alone, (ii) with anti-mouse antibody and Protein A-Sepharose and (iii) mAb11C7 and Protein A-Sepharose.

Immunogold-labeling and transmission electron microscopy

The procedure for the preparation of whole mount cytoskeletons of *T. brucei* on EM grids are described in detail elsewhere (Mueller et al. 1992, Rindisbacher et al. 1993). All subsequent steps were carried out at 24°C. Fixation of cytoskeletons was performed in 3% paraformaldehyde/0.1 % glutaraldehyde in MME-buffer for 30 min, and blocking of unspecific binding sites was carried out with blocking solution for 40 min (see above). Then the mAb11C7 was applied at a dilution of 1:1 in blocking buffer for 40 min, followed by several rinses in PBS. The second antibody conjugate was a goat anti mouse IgG conjugated to 10 nm gold particles (Amersham). This was used at a dilution of 1:5 in blocking buffer for 40 min. Finally the grids were washed 6 times 5 min in PBS, and were fixed in 2% glutaraldehyde in PBS for 30 min. The specimens were then rinsed several times in distilled H_2O and were negatively stained with 1% uranyle acetate as described (Mueller et al. 1992, Rindisbacher et al. 1993).

For preembeddment labeling of *T. brucei* cytoskeletons with mAb11C7, cytoskeletons (five times 10^7) were fixed in 2 ml of MME containing 3% pFA and 0.1% glutaraldehyde for 30 min at 4°C. They were then washed twice in PBS by centrifugation and were treated with 50 mM glycine in PBS for 30 min in order to block free aldehydes. Cytoskeletons were then incubated in blocking solution for 30 min, and were subsequently labeled with mAb11C7 (diluted 1:1 in blocking solution) for 1 h. After two washes in PBS, the second antibody was applied at a dilution of 1:5 in blocking solution. Cytoskeletons were then washed in PBS and were fixed in 2% glutaraldehyde in 100 mM sodium phosphate buffer pH 7.2 for 4 h at 4°C. Cytoskeletons were washed in phosphate buffer, postfixed in 2% $0s0_4$, dehydrated in a graded series of ethanol and embedded in Epon 812 for 48-72 h. The preparations were polymerized at 65°C for 24-48 h. Thin sections were cut with a diamond knife using a Reichert and Jung ultramicrotome, and the grids were stained with lead citrate and uranyle acetate. All preparations were observed using a Jeoel 100 CX II transmission electron microscope operating at 60-80 kV.

RESULTS AND DISCUSSION

Ten monoclonal antibodies directed against *Tetrahymena* tetrins were tested for their ability to recognize *T. brucei* cytoskeletal proteins in Western blot analyses. mAb11C7 produced a strong reaction with a 300-kDa doublet, as shown in Fig. 1; mAbs2D3 and 12F3 gave a relatively weak reaction with a band at the 120 kD position (not shown). MAbs4G9, 2D10, 8C10, 9E9, 4B3, 1H7 and 21A5 did not recognize *T. brucei* proteins in these tests. All ten monoclonal antibodies were used in immunofluorescence recognition tests with

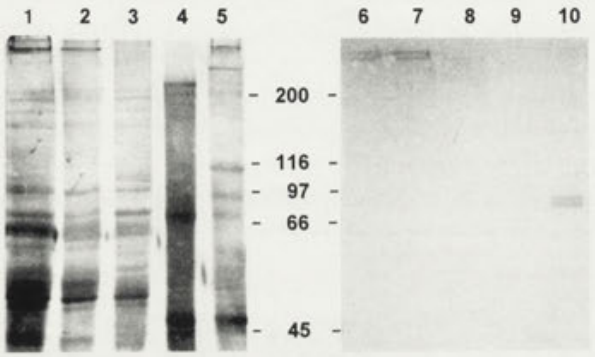

Fig. 1. Immunoblot showing recognition of *Tetrahymena* and *Trypanosoma* proteins by the anti-tetrin 4-specific mAb11C7. Proteins separated in 6% polyacrylamide gel and stained with Coomassie-blue are shown in lanes 1-5. Lanes 6-10 are the corresponding lanes from the nitrocellulose transfer filter after probing with mAb11C7 and an alkalinephosphatase conjugated anti-mouse IgG. The preparations are *T. brucei* procyclic total cell protein (lanes 1, 6), *T. brucei* Triton X-100 insoluble residues (lanes 2, 7), *T. brucei* Triton X-100 soluble fraction (lanes 3, 8), human red blood cell ghosts (lanes 4, 9) and *Tetrahymena* cortical residues (lanes 5, 10) The 11C7 antibody recognized a band at 80 kDa (arrowhead) in the *Tetrahymena* cortical cytoskeleton as expected, and a doublet at about 300 kDa (arrow) in the *Trypanosoma* cytoskeletal fraction. The band at 160 kD are tetrin4-dimers. The molecular weight markers were myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa)

T. brucei whole cells and Triton X-100 extracted cytoskeletons. Only mAb11C7 was positive (Figs. 3, 4). This antibody was selected for further study.

Red blood cell proteins were included in the immunoblot in Fig.1 as an additional check on the size of the HMW-proteins recognized by mAb11C7 in *T. brucei*. It is seen in the first panel that the latter are somewhat larger than spectrin. Spectrin is the top band in lane 4 and has a molecular weight of 240 kDa. *Tetrahymena* tetrin 4 has a molecular weight of about 80 kDa (lane 10)

Knowing that *Tetrahymena* tetrins are phosphoproteins (N. E. W., unpublished), we decided to see whether or not a phosphorylated 300-kDa protein could be immunoprecipitated from *T. brucei* using mAb11C7. The cells were first labeled with carrier-free ^{32}P, then detergent extracted and sonicated. The resulting extracts

were reacted with mAb11C7 and immunoprecipitated with anti-mouse antibody and Protein A Sepharose. The precipitates were subjected to electrophoresis and examined by autoradiography. As shown in Fig. 2, a radioactive band was found at the molecular weight position of the 300 kDa mAb11C7-reactive proteins of *T. brucei*. This raises the possibility that phosphorylation and/or dephosphorylation may regulate the assembly competence or other biological activity of this protein in *T. brucei*.

The mAb11C7-positive high molecular weight proteins in *T. brucei* are much larger than *Tetrahymena* tetrin 4, against which the mAb11C7 was raised. No explanation for this can be offered at the present time.

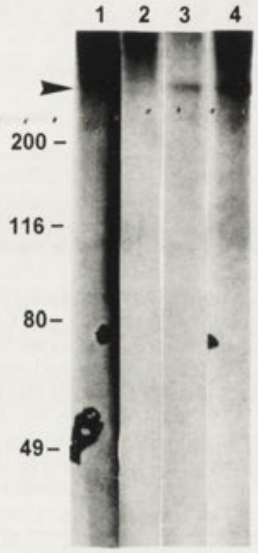

Fig. 2. Recovery of a 300-kDa phosphoprotein from *T. brucei* by immunoprecipitation with anti-tetrin mAb11C7. *T. brucei* procyclics were labeled with carrier-free ^{32}P. The cells were extracted with Triton X-100 and the cytoskeletons collected and sonicated. This extract was reacted with Protein A-Sepharose (lane 1), anti-mouse antibody and Protein A-Sepharose (lane 2), mAb11C7 and Protein A-Sepharose (lane 3), and mAb11C7 plus anti-mouse antibody and Protein A-Sepharose (lane 4). A heavily phosphorylated band was found at the 300-kDa position in autoradiographs of the 11C7 immunoprecipitates (arrow), but not in the controls. The molecular weight markers were myosin (200 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (80 kDa), and fumarase (49 kDa)

Related proteins with substantially different molecular weights are not unknown (Albanesi et al. 1985). On the other hand, immunological crossreactivity alone is not enough to establish evolutionary relationships., however. Only detailed studies of the *Tetrahymena* and *Trypanosoma* proteins themselves, or the genes that specify them can resolve the question of the precise nature of their relationship.

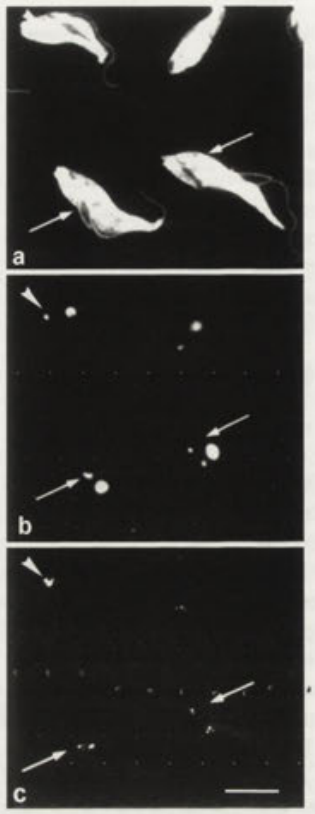

Fig. 3. Localization of tetrin-like proteins in *T. brucei* by triple staining cells with anti-tubulin (**a**), DAPI (**b**), and mAb11C7 (**c**). As indicated by the arrows, there are three tetrin-reactive dots (**c**) at the base of each flagellum (**a**) immediately adjacent to the kinetoplast (smaller of the stained bodies in **b**). A non-divider is indicated by the arrowhead, and dividing cells are indicated by arrows. Bar - 2μm

Immunofluorescence tests of *T. brucei* cells and cytoskeletons using mAb11C7 revealed the presence of three small but very bright spots near the posterior end in nondividing cells (Figs. 3c, 4c) Triple labeling was used to more precisely determine the location of the tetrin dots in relation to other structures within the cell. *T. brucei* cells and cytoskeletons were stained with an anti-tubulin antibody to show the cortex and flagellar structure (Figs. 3a, 4a), with DAPI to reveal the positions of nuclei and kinetoplasts (Figs. 3b, 4b), and with mAb11C7 to show the location of the high molecular weight proteins (Figs. 3c, 4c). Each cluster of tetrin dots in these preparations was found to lie very close to the kinetoplast and seemed most likely to be associated with the base of the flagellum. The regular occurrence of dots in clusters of three is intriguing, but without explanation at present.

Transmission electron microscopy was used to confirm the presence of these proteins at the base of the flagellum in *T. brucei*. Whole mount preparations on grids were treated with mAb11C7 and gold-conjugated

Fig. 4. Localization of tetrin-like proteins in a dividing *T. brucei* cell. The cell is visualized with anti-tubulin in (**a**), DAPI in (**b**), and mAb11C7 in (**c**). Three tetrin dots are seen adjacent to each daughter kinetoplast at the base of each flagellum. Bar - 2μm

anti-mouse antibodies. As shown in Fig. 5c, gold particles were found at the periphery of flagellar basal bodies. *T. brucei* cytoskeletons were also treated with mAb11C7 and gold-conjugated second antibody, embedded and sectioned. Examination of these preparations also revealed the presence of gold particles attached to filamentous material surrounding the flagellar basal bodies (Figs. 6, 7). Exactly the same distribution was observed in cytoskeletons embedded in LR-Gold

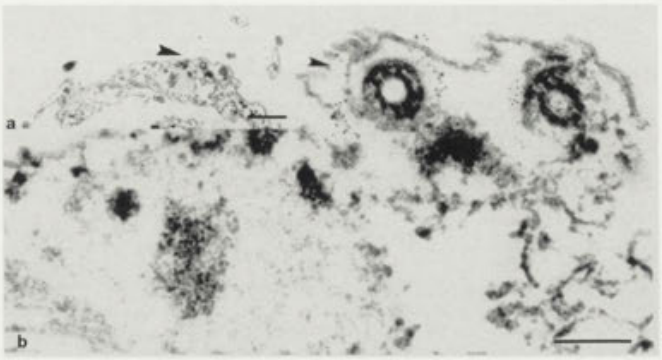

Fig. 6. Thin section of a *T. brucei* cytoskeleton stained with mAb11C7 and goldconjugated anti-mouse antibody prior to embedding. **a** - low power view showing location of the two basal bodies (arrow), bar - 900 nm. **b** - higher magnification showing gold particles adjacent to both basal bodies, bar - 220 nm

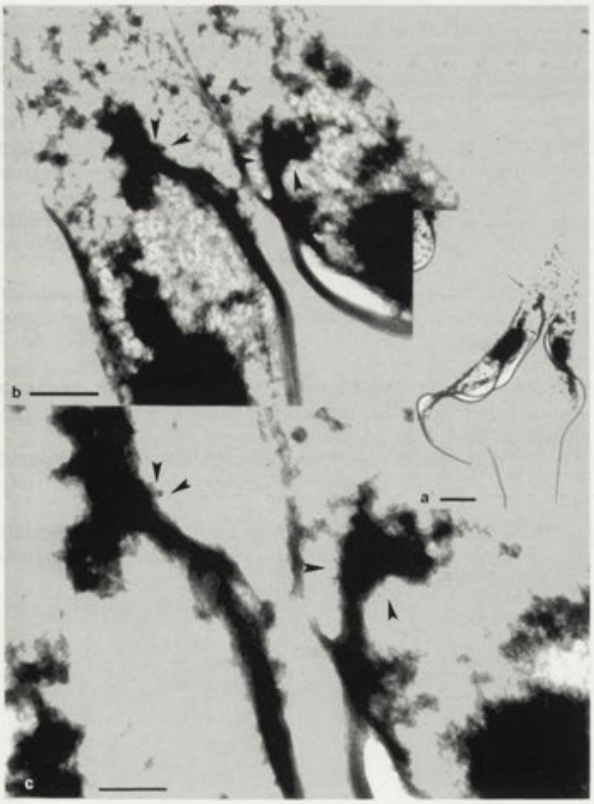

Fig. 5. Negatively stained whole cytoskeletons of *T. brucei* showing that the tetrin-like proteins are associated with the flagellar bases. The preparations were stained on the grids with mAb11C7 and 10 nm gold particles conjugated to anti-mouse antibodies. **a** - low magnification showing entire detergent-insoluble residues, bar - 1 μm. **b** - higher power showing gold particles (arrows) associated with the flagellar bases, bar - 360 nm. **c** - high magnification showing gold particles (arrows) surrounding the basal bodies, bar - 210 nm

and stained following sectioning, although the number of gold particles per section was low (not presented).

Anti-tetrin mAb11C7 was also tested both in Western blots and in fluorescence preparations against a variety of trypansosomes and life cycle stages. A 300-kDa doublet band was recognized in immunoblots of cytoskeletal proteins from *T. brucei* (bloodstream forms and procyclics), *T. congolense* (bloodstream forms and epimastigotes), *T. vivax*, and *T. evansi*. Tetrins were found in immunofluorescence preparations of all cell types. Neither tetrin dots nor recognition in immunoblots was found in *T. cruzi* (amastigotes, trypomastigotes, epimastigots), *Leishmania major* (promastigotes), *L. tropica* (promastigotes), *L. infantum*, *L. mexicana*, or *L. brasiliensis*. The overall result is that mAb11C7-reactive proteins were found in the African trypanosomes by these procedures, but not in the South American trypanosomes. However, it is possible that the epitope recognized by mAb11C7 may be present, but inaccessible, in the South American forms. This can only be resolved by further studies.

The results presented above suggest that the high molecular weight proteins recognized by mAb11C7 are present in African trypanosomes at all stages in the life cycle. These polypeptides are associated with basal bodies, as are the tetrins in ciliates. However, future studies of these proteins may contribute to our understanding of the organization, function and biogenesis of basal bodies in these microbial eukaryotes.

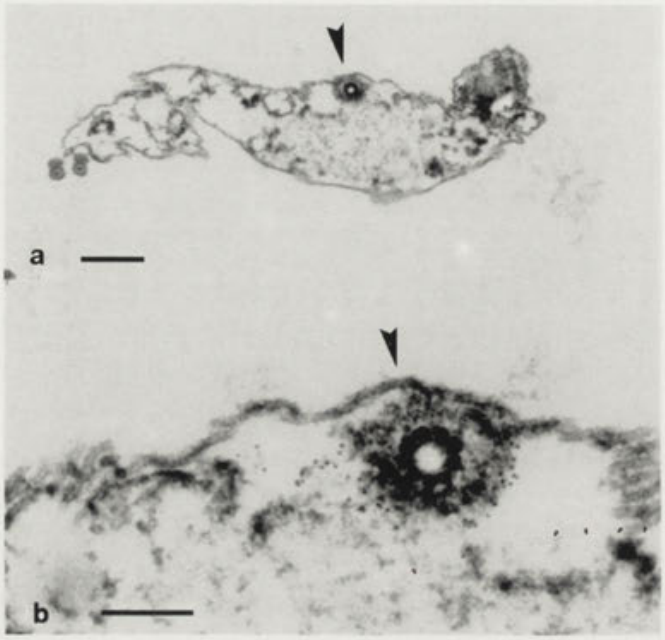

Fig. 7. Thin section of a *T. brucei* cytoskeleton showing the presence of tetrin-like proteins in the dense material surrounding the basal body (arrows). The cytoskeletons were treated with mAb11C7 and gold-conjugated second antibody prior to embeddment. **a** - low magnification, bar - 650 nm. **b** - high magnification, bar - 185 nm

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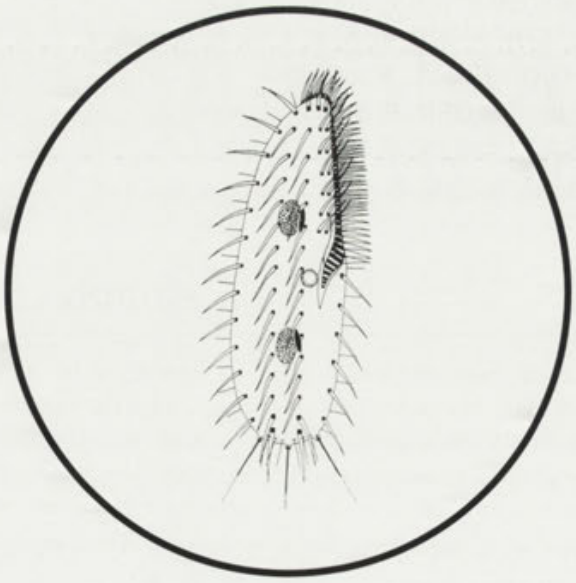

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