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Review article

Advanced Methods in Photobiology of Protists

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INTRODUCTION

The field of photobiology in protists covers such diverse topics as orientation of microorganisms (photomovement), movement of intracellular organelles (photodinesis) or light-dependent development (photomorphogenesis). Though many of the basic phenomona have been discovered more than a century ago (Cohn 1863, Engelmann 1882, Aderhold 1888, Jennings 1904) most earlier studies were restricted to phenomenological description due to the limited availability of suitable research tools.

Photomovement was studied by following tracks of individual cells, which was hampered by time consuming microscopic analysis. However, it is remarkable how accurate and painstakingly early researchers observed the behavior of the organisms in spite of the limitations of their optical instruments. Likewise, the orientation of intracellular organelles was investigated manually (see reviews Haupt 1983, Haupt and Wagner 1987).

Lateron, microscopic observation was supported by population techniques in which the movement of a whole population of microorganisms was followed using devices such as photocells which analyzed the change in cell density when the organisms swam unidirectionally, e.g., toward the light source (Nultsch 1962, Diehn 1969, Feinleib 1975). Similar photometrical techniques were applied to the research of intracellular movement (Nultsch and Pfau 1979).

The observation of photoinduced responses arose theoretical interest in the information transmission between the photoreceptor and the final behavioral or developmental response, called sensory transduction (Nultsch 1991, Haupt 1991). This chain of events involving spatial transmission, energy conversion and hierarchical evaluation of diverse stimuli was regarded as a black box (Colombetti and Marangoni 1991) and elucidated by indirect methods such as action spectroscopy or the application of drugs (see reviews Häder 1979a, Nultsch and Häder 1979, 1988). The identification of a chromophore as a photoreceptor by comparison of the action spectrum for a given response with the known absorption spectrum of isolated pigments has to be regarded as preliminary, since a number of factors such as shading pigments, occlusion by other bulk pigments or wavelength shifts due to protein binding pose numerous problems (Song 1991, Lenci 1991). Likewise, most drugs such as electron transport inhibitors or uncouplers of the energy conversion steps, applied for a specific action are known to

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impair other cellular functions as well (Nultsch 1975). However, a number of ingenious experiments designed with simple methods have allowed some insight into the sensory transduction chain. E.g., the simple observation of flagellates swimming toward the light both in a converging and a diverging light beam proved that these organisms actually perceive the light direction rather than orient with respect to a light intensity gradient (Buder 1919). Also the experiments using polarized light to demonstrate the birefringence of the absorbing vectors of photoreceptor pigments allowed new insight into the mechanism of photoperception (Haupt and Schönbohm 1970).

Today the observation of behavioral responses of cells and organelles is facilitated by video and computerized image analysis (Hand and Davenport 1970, Feinleib and Curry 1967, Watanabe and Furuya 1982, Boscov and Feinleib 1979, Davenport et al. 1970). Fast responses can be analyzed by laser light scattering and high speed microscopy. Advanced spectroscopic techniques such as fluorescence life time determination or time-gated fluorescence studies allow to reveal the molecular events taking place during excitation and relaxation of photoreceptor molecules. Advanced electrophysiological methods such as patch clamp or intracellular microelectrodes help analyze membrane bound electrical events within the sensory transduction. And even space biology is utilized to understand the cellular events triggered by light and other stimuli. Recently developed biochemical techniques such as SDS PAGE, IEF, 2D gel electrophoresis, immunological methods and FPLC help to understand the molecular basis of photoperception, sensory transduction and modulation of the response. Molecular genetics is also being employed to reveal the identity of gen products involved in the cellular information exchange both in photomovement and in photomorphogenesis. These techniques are the topic of this review article, however, the selection of specific examples will be biased and the stress on certain aspects will be influenced by the interests of the author.

IMAGE ANALYSIS

The availability of powerful hardware components and the development of effective algorithms (Sasov 1989) has facilitated the construction of computerized image analysis systems which found widespread use in many biological and medical sciences (Häder 1988, Häder and Vogel 1991a, Frank 1989, Dhawan 1990, Kenny et al. 1990). Applications in the photobiology of protists range from counting cells or colonies over area measurements in cells and organelles to the automatic tracking of moving organisms. In addition, the development of confocal microscopy, atomic force microscopy and other digital techniques helps reveal cellular structures *in vivo* at a resolution which was limited to electron microscopical techniques of fixed material. In the following the basic hardware and software components are briefly reviewed and their application demonstrated in a few examples.

Hardware configuration

The source of the image to be analyzed can be a video or CCD (charged coupled device) camera, a video recorder or a scanner such as a laser scanner for two-dimensional gels. The magnification is determined by the size of the object, which can range from microscopical to macroscopical. A system to track photomotile protists consists of a dark field microscope (in order to enhance the contrast) equipped with a CCD camera which constantly records the image of the moving cells on line (Fig. 1). The monitoring beam is in the infrared in order to avoid interference with the photoresponses to be studied and to exclude the production of photosynthetically generated oxygen which is known to induce a pronounced aerotaxis in some organisms.

The first step in image analysis is the digitization of the analog image. First the image is divided into an array of discrete image points (pixels), the number of which (e.g., 512 x 512) determines the spatial resolution. Each pixel is assigned a digital gray value (e.g., in the range of 0 = dark black to 255 = bright white) (Preston 1986, Desai and Reimer 1985) which is stored in an electronic memory which can be on board of the digitizer itself or located in the main computer memory (Danielsson and Kruse 1980, Steinbach et al. 1982, Allen and Allen 1983). In older instruments his analog to digital (A/D) conversion was performed sequentially for all image points and was thus rather time consuming. Modern digitizers or frame grabbers are equipped with flash converters and perform this task in real time, i.e., in 40 ms per full frame (European video standard) or 33 ms (US video standard). Thus, an incoming video sequence from a camera can be digitized on line (stream modus); but this does not necessarily mean that it is analyzed at the same speed. While only a few years back digitizers were stand alone instruments they have shrunk to a single plug-in card which fits into an AT type microcomputer. For most

applications it is sufficient to take a snapshot of the scene which is analyzed subsequently. For most applications a b/w video signal is sufficient and only under certain conditions a color signal is necessary which requires parallel digitization of all three color channels (red, blue, green) (Castillio et al. 1982). Also the required memory space triples in color processing.

Most frame grabbers allow the adjustment of brightness and contrast by software control before A/D conversion. Furthermore, the image is manipulated before it is stored in memory by using one of several selectable look-up tables (LUT, Bryan et al. 1985): for each digitized value of the original image a new one is looked up in a previously defined table in order to, e.g.,

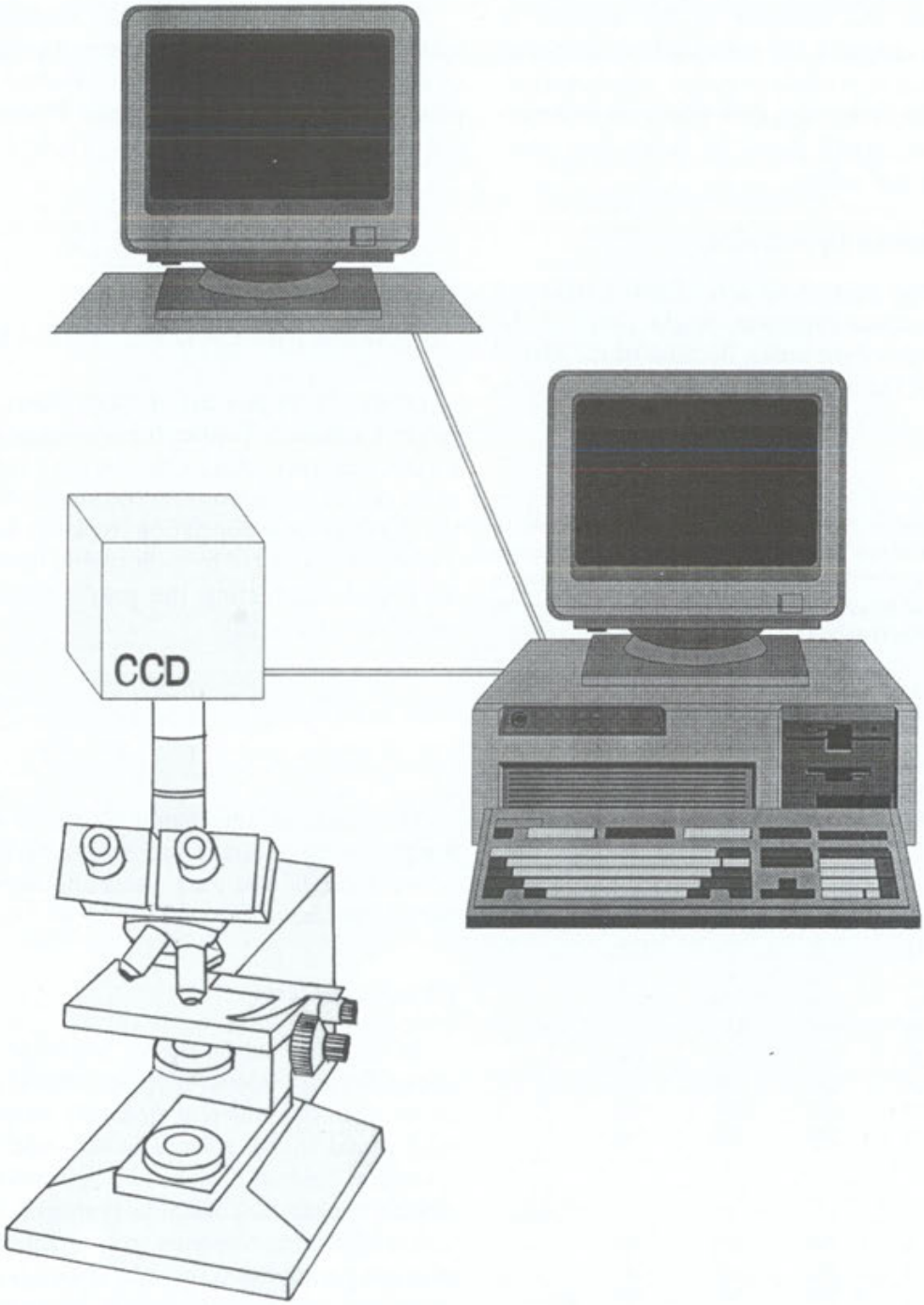

Fig. 1. Schematic representation of an image analysis system to track protists. The organisms move in a cuvette on the stage of a dark field, infrared microscope and the image of the moving organisms is recorded by a CCD camera, the signal of which is digitized by a frame grabber inside an IBM AT compatible microcomputer. A separate analog color monitor shows the digitized image (after Häder and Vogel 1991)

enhance the contrast, reduce background noise or electronically invert the image (Table 1).

The host computer has access to the image in memory and can both read and write individual pixels, thus analyzing and manipulating the image (Kemnitz and Hougardy 1980, Russ and Russ 1984, Mayfield 1984). The result of digitization and manipulation is visualized on a video monitor after D/A conversion. For this purpose the contents of the memory is compared to an output LUT which allows to assign certain gray values to predefined colors, thus constructing a pseudocolor image: e.g., dark pixels are represented by blue hues, bright pixels by green and intermediate ones by red colors.

Image manipulation by software

Table 2a shows part of an array from a digitized image. High numbers represent bright parts of the image and low ones dark areas. Because of the digital representation of the image it can be easily manipulated using simple mathematical filter techniques (Coltelli and Gualtieri 1990, Marangoni et al. 1991). By an addition or subtraction of a constant from the gray value in each pixel the image can be made brighter or darker. Vertical or horizonal gradients allow to correct for areas of uneven brightness within the image. The contrast can be enhanced by multiplying each gray value with a constant.

Smoothing is also a simple standard technique which is based on the calculation of arithmetic means of neighboring pixels (Julez and Harmon 1984): each pixel P is replaced by the mean P' calculated from a 3 x 3 (or larger) pixel matrix (Table 2b).

$$\begin{matrix} A & B & C \\ D & P & E \\ F & G & H \end{matrix}$$

$$P' = (P+A+B+C+D+E+F+G+H)/9 \qquad (1)$$

Other algorithms are median filters, Sobel algorithm, Laplace or Fourier transformations with which certain features such as edges between bright and dark areas can be extracted from the image. As an example, the Laplace transformation replaces each pixel by a new one calculated by multiplying the original value by 8 and subtracting the gray values of all eight neighbors (Table 2c).

$$\begin{matrix} -1 & -1 & -1 \\ -1 & 8 & -1 \\ -1 & -1 & -1 \end{matrix} \qquad (2)$$

The result of this manipulation is that uniform (bright or dark) areas are set to dark while edges between bright and dark areas appear as lines (compare Table 2c).

Object counters

In order to recognize an object in the digitized image, the gray values (or color codes) of its pixels need to be distinctly different from the background. This may sound trivial: when a human operator observes a scene of colored protists in a light microscope, dark objects appear on a bright background. However, our visual apparatus responds to contrast differences and may not perceive that the field of view is not irradiated uniformly and that the bright background in some areas is actually even darker than the organisms in another area. An automatic image analysis system has

Table 1

Use of look-up tables, to invert an image into its negative (A), to reduce the gray scale to two steps in contrast (B) or to stretch a small range of gray values over the whole range. Pseudocolor representation can be achieved by assigning specific gray values to one of the three color channels (D) (after Häder 1988)

	A	B	C	D	
0	255	0	0	0	
1	254	0	0	3	
2	253	0	0	6	blue
3	252	0	0	9	
4	251	0	0	12	
.	.	.	.	.	
.	.	.	.	.	
.	.	.	.	.	
124	131	0	31	118	
125	130	0	63	121	
126	129	0	95	124	
127	128	255	137	127	red
128	127	255	169	130	
129	126	255	201	133	
130	125	255	233	136	
131	124	255	255	139	
.	.	.	.	.	
.	.	.	.	.	
.	.	.	.	.	
251	4	255	255	243	
252	3	255	255	246	
253	2	255	255	249	green
254	1	255	255	252	
255	0	255	255	255	

Table 2a

Part of an array from a digitized image. Low values indicate dark image points (pixels), high values bright ones. Note the high value (140) in the second row, third columm which is a flaw of a bright pixel in a dark surrounding (after Häder 1988)

60	52	61	63	75	71	68	72	78	81	79	84	85
61	64	140	72	75	71	79	80	85	81	84	89	92
58	61	64	67	80	70	72	75	78	80	78	82	85
64	71	72	75	71	69	220	231	225	228	229	231	230
65	70	58	73	75	230	229	228	234	227	230	232	218
69	72	75	73	225	247	230	231	235	237	228	229	240
71	75	72	74	68	242	250	231	232	232	228	237	239
79	69	72	75	76	71	228	229	235	235	236	228	227
60	63	65	71	72	72	228	229	230	225	230	230	225
63	70	68	72	74	73	230	229	231	234	234	230	228

Table 2b

Noise reduction and smoothiug by averaging over a 3 x 3 matrix in table 2a

60	52	61	63	75	71	68	72	78	81	79	84	85
61	69	72	77	72	73	73	76	79	80	82	84	92
58	73	76	80	72	90	107	127	129	130	131	133	85
64	65	68	71	90	124	158	177	178	179	180	179	230
65	68	71	89	126	177	213	229	231	230	230	230	218
69	70	71	88	145	200	235	233	232	231	231	231	240
71	73	73	90	128	182	218	233	233	233	232	232	239
79	70	71	72	91	145	198	232	231	231	231	231	227
60	68	69	72	73	125	177	230	231	232	231	230	225
63	70	68	72	74	73	230	229	231	234	234	230	228

Table 2c

Result of Laplace filtering with a kernel of 8 using values in table 2a

60	52	61	63	75	71	68	72	78	81	79	84	85
61	0	255	0	31	0	53	33	55	5	18	43	92
58	0	0	0	70	0	0	0	0	0	0	0	85
64	56	37	40	0	0	255	255	255	255	255	255	230
65	14	0	0	0	255	146	0	30	0	0	21	218
69	21	33	0	255	255	0	0	28	50	0	0	240
71	21	0	0	0	255	255	0	0	0	0	41	239
79	0	12	30	0	0	255	0	37	32	43	0	227
60	0	0	0	0	0	255	0	0	0	0	2	225
63	70	68	72	74	73	230	229	231	234	234	230	228

problems with such a situation when it discriminates the organisms from the background by using a simple thresholding technique; therefore specific care needs to be taken to warrent a homogeneous irradiation.

Figure 2 shows a digital image with a number of protists to be counted. Since we are interested only in the number of cells and not in cellular details it suffices to regard the image as binary: each pixel has only two states, background or organism. The threshold between the two can be found empirically or by determining a histogram of the gray level distribution (Fig. 3) : the small left peak represents the few dark cell pixels and the large right peak the bright background pixels. The threshold is defined between the two peaks.

The search algorithm starts by scanning the image row by row until it finds a first object pixel, the position of which is stored. Now all (eight) neighbors are queried whether or not they are also object pixels; in this case they belong to the same object by definition and are set to a reserved gray (color) value. This search

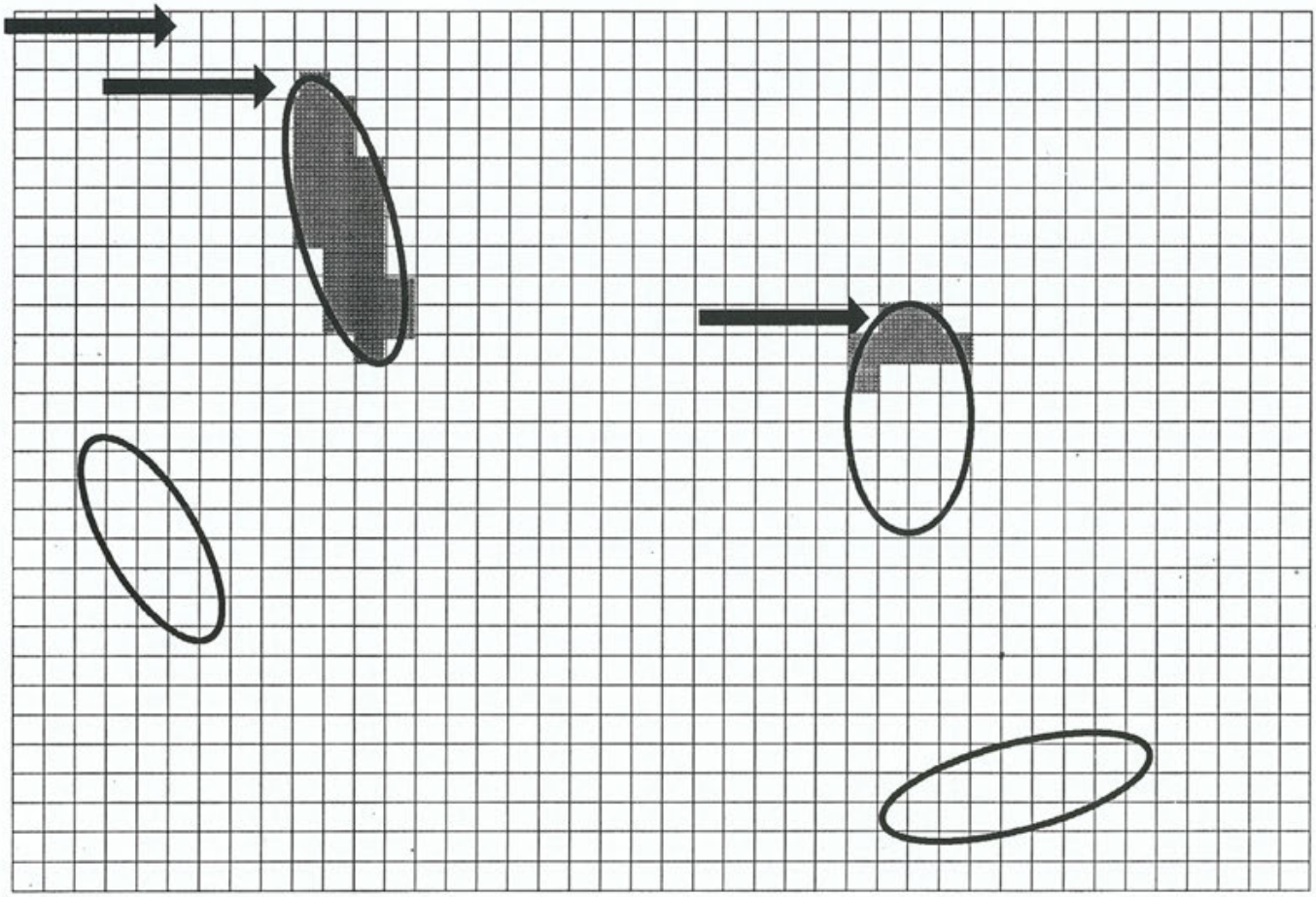

Fig. 2. A digital image with a number of protists to be counted. The search algorithm starts scanning from the top left hand corner row by row (arrows) until it detects a pixel distinctly different from the background by a predefined threshold (Häder and Vogel 1991)

is repeated iteratively until all pixels of this object are found. Afterwards the scan is continued from the first found object pixel until the image is completed. All cells already found are recognized as such because of the reserved gray value their pixels are set to.

This technique was used to study the light-induced vertical movements of flagellates in several ecological studies both in freshwater and marine habitats (Häder and Griebenow 1988, Eggersdorfer and Häder 1991a, b). A large Plexiglass tube (3 m long, 70 mm inner diameter) was filled with a population of phytoplankton organisms and thermally equilibrated. Light was applied from above simulating a natural summer day. In order to determine the vertical movement of the organisms over the day, samples were drawn at regular time intervals from 18 outlets evenly spaced along the length of the column using a peristaltic pump with 18 parallel positions. It is obvious that manual counting would have been tremendously time consuming specifically using frequent sampling time intervals. Therefore the samples were drawn automatically into a cuvette on the stage of a microscope by another peristaltic pump and the cell density was determined using the image analysis system described above equipped with the count program covered in the last paragraph (Häder and Griebenow 1987). In order to increase the statistical significance, several replicates were measured for each sample. Figure 4 shows the vertical density distribution of a marine dinoflagellate population over a period of 38 h. Similar vertical migrations were found in a number of dinoflagellates both in tank experiments and open water studies (Yentsch et al. 1964, Taylor et al. 1966, Tyler and Seliger 1978, 1981, Estrada et al. 1987, Holmes et al. 1967).

Size and area determination

The fill algorithm described above is relatively slow since all pixels in an organism need to be queried. Therefore the faster Chain Code Algorithm is applied

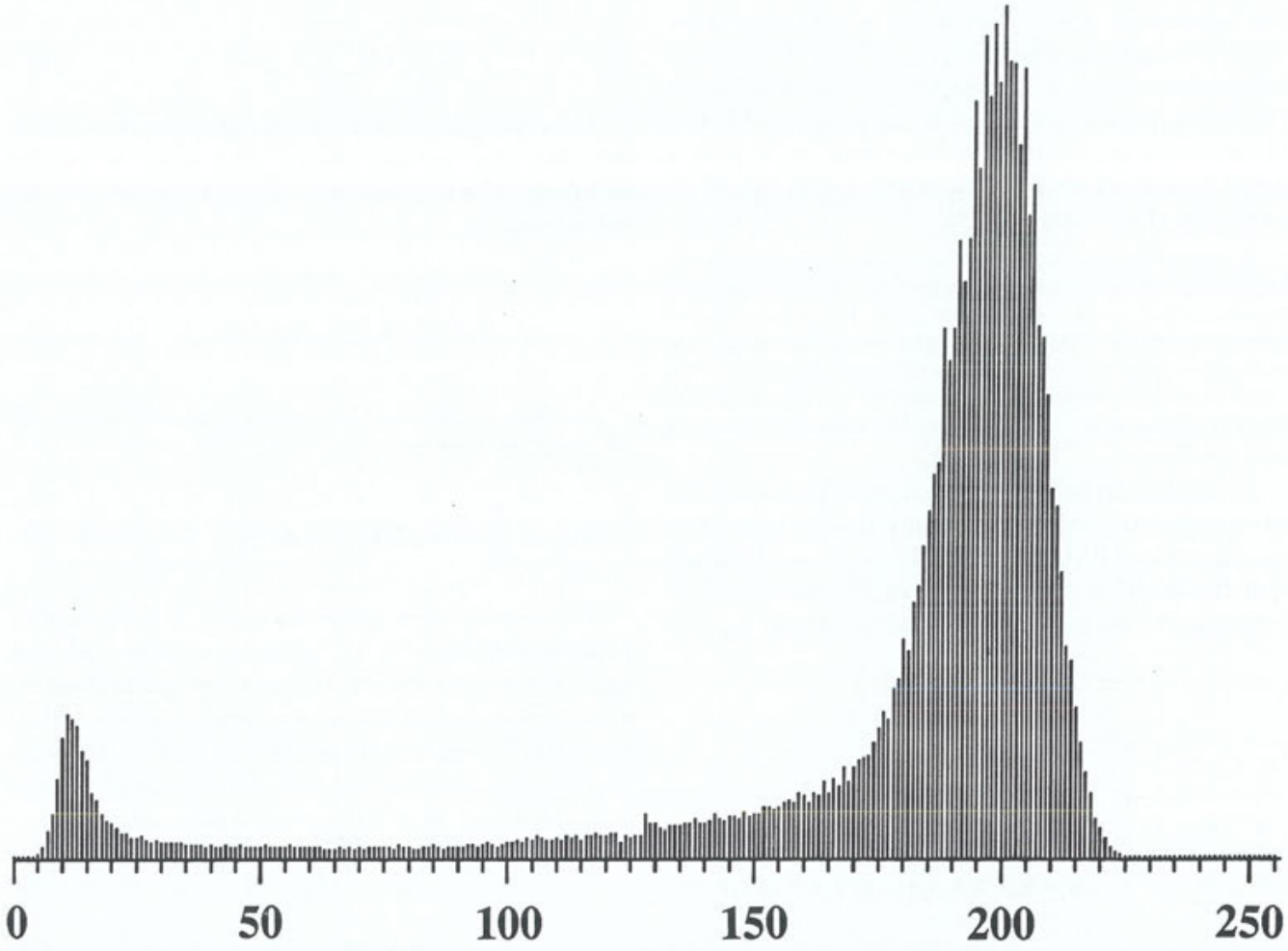

Fig. 3. Histogram of the gray level distribution in a digital image; the small peak on the left represents the few dark cell pixels and the large peak on the right the bright background pixels

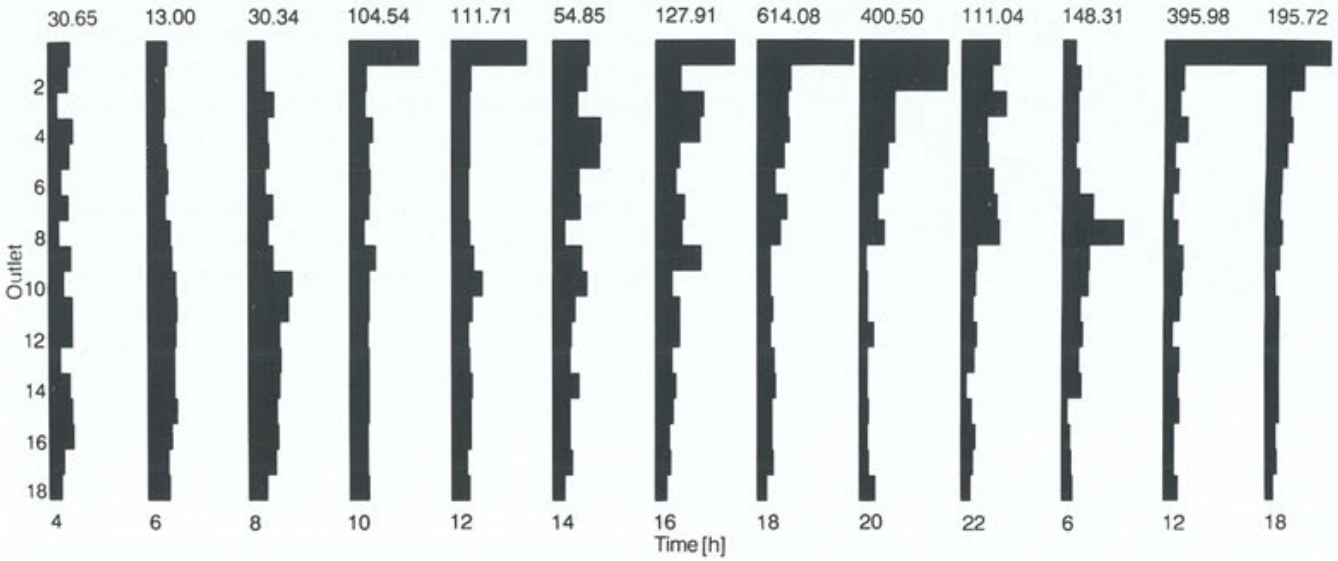

Fig. 4. Vertical cell density distribution of the marine *Peridinium faeroense* in a 3 m long Plexiglass column under simulated daylight conditions

to determine the area of larger objects (Freeman 1961). The initial edge pixel of an object is again found by sequential scanning of rows of pixels. Once this starting point is determined, only edge pixels are searched for in a clockwise manner until the starting point is hit again. The direction vector from one edge pixel to the next is coded by a number between 0 and 7 and stored in a growing chain code (Fig. 5).

In order to find the next edge position on the circumference, the neighboring pixels are tested starting from the previous scan direction shifted 2 positions counterclockwise. This is performed by a modulo 8 addition of 2 to the last direction vector. If this pixel is not an organism (edge) point the shift is decremented (modulo 8) until a valid edge pixel is found. As an example the first edge point was found by moving to the lower right; this first chain code element is defined as 7.

In addition to being a very fast algorithm it has the advantage that mathematical analysis techniques have been developed (Freeman 1974, 1980). From the chain code the length L of the outline can be calculated by summing the even numbered chain elements a_{ie} plus the odd numbered ones a_{io} times the square root of 2

$$L = T(a_{ie} + a_{io}\sqrt{2}) \quad (3)$$

The scaling factor T allows to adjust the pixel size to the real size in the image. The area A of the two-dimensional projection of the organism can be determined by

$$A = \sum_{i=1}^{n} a_{ix}(y_{i-1} + 1/2\, a_{iy}) \quad (4)$$

In order to calculate the center of gravity (centroid) we need the first moments about the x axis:

$$M_{1x} = \sum_{i=1}^{n} 1/2\, a_{ix}[(y_{i-1} + a_{iy}(y_{i-1} + 1/3\, a_{iy})] \quad (5)$$

The first moment about the y axis is computed by rotating the chain by $\pi/2$ about the origin and then again using equation (5). The rotation can be done by rotating the coordinates of the origin and then adding 2 modulo 8 to each chain link. Using the first moments and the area, the centroid with the coordinates x_c and y_c is calculated by the following equations

$$x_c = -M_{1y} / A \quad (6)$$

$$y_c = M_{1x} / A \quad (7)$$

During the chain code determination all edge pixels are arbitrarily set to 255 (bright white) and serve as markers for already found organisms. Starting from the first found edge pixel of the previously calculated organism the search is continued until the whole frame has been analyzed and all organisms have been found.

Problems occur when adjacent objects touch or partially occlude each other. Some of these problems can be solved by erosion and dilation techniques (Erhardt et al. 1980, Bovik et al. 1991): shells one pixel wide are removed from the objects iteratively until the isthmus between touching objects is separated. Then the individual objects are blown up (dilated) again to their former size and shape leaving a gap at least one pixel wide between cells.

3	2	1
4	P	0
5	6	7

Chain Code:

7,0,6,6,6,6,5,5,5,4,4,5,4,5,4,5,4,

5,4,3,1,2,1,1,1,1,0,1,1,1,0,1,1

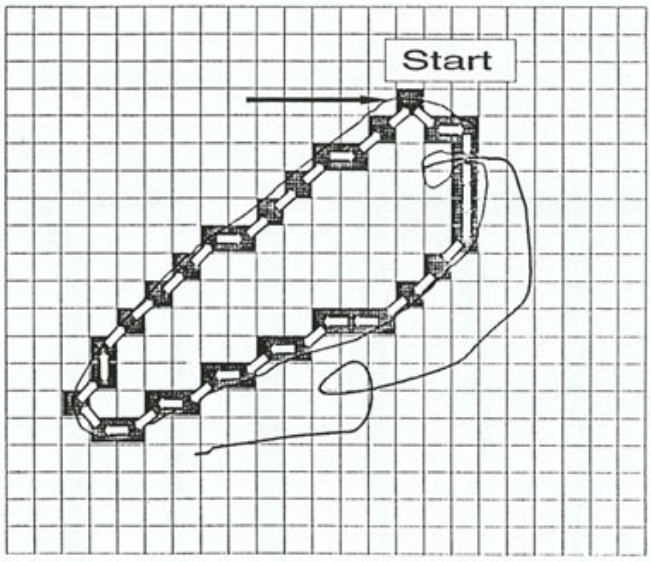

Fig. 5. Demonstration of the Chain Code algorithm using the outline of a *Euglena* cell. From the start point the vectors are determined to the next edge pixel in a clockwise fashing and coded according to the scheme shown in the top left (Häder and Vogel 1991)

Tracking

Studying the photomovement of individual cells is greatly facilitated by using automatic tracking systems

based on computerized image analysis. These systems allow the analysis of large numbers of cells at high precision and speed. Earlier systems recorded sequences of video frames for subsequent frame by frame analysis in order to follow the movement vectors of organisms in the time domain (Allen 1985, Dow et al. 1987, Amos 1987, Davenport et al. 1970, Davenport 1973, Coates et al. 1985, Rikmenspoel and Isles 1985, Burton et al. 1986). Likewise, the growth of an organism or organelle can be followed using these techniques (Gordon et al. 1984, Omasa and Onoe 1984, Jaffe et al. 1985, Omasa and Aigi 1987, Popescu et al. 1989). The tracked objects can range from individual cells such as flagellates or ciliates (Mikołajczyk et al. 1985, 1986, Häder et al. 1986, 1987, 1988, Musgrave and Häder, 1991) to multicellular or macroscopic organisms (Dusenbery 1985, 1991). Parameters such as directionality, velocity or percentage of motile organisms in a population can be recorded in a population. Even the movement of a single flagellum or cilium can be determined (Sanderson and Dirksen 1985, Baba and Mogami 1985, Omoto and Brokaw 1985, Cantatore et al. 1989). After an object (or all objects in the frame) has been identified by using either of the techniques described above it is prudent to determine whether or not it is likely to be an organism by using a number of comparisons. Upper and lower limits for the area allow to discriminate a valid organism from debris. Also the contour length or form factors such as the ratio between the contour length to the area can be employed as additional classification factors.

In an earlier approach to track microorganisms (Häder and Lebert 1985) one organism was identified in the image using a random number algorithm and the coordinates of its baricenter were stored. After digitizing the next frame from the video sequence, the new position of this organism was determined starting from the baricenter in the previous frame. If this was not found to be in the new outline of the cell, it was regarded as lost.

Another technique operates by repetitive subtraction of video images from the initial one (Gualtieri et al. 1985, Gualtieri and Coltelli 1991). All non-moving parts (background and immotile organisms) are thus removed from the screen and only moving objects are kept. By repetitive subtraction the tracks of all objects appear as wide lines which are skeletoned in a subsequent step until the tracks are represented by lines one pixel wide. Finally, the movement vectors of all cells are extracted from the resulting image. A similar system is used to track *Chlamydomonas* and other flagellates (Kondo et al. 1988, Watanabe 1991). After analysis the resulting tracks are printed on a color printer; subsequent track segments are color coded which allows to reconstruct individual paths.

In order to analyze the movement of protist with respect to light and other stimuli in real time a new computerized image analysis system was developed using assembly language modules since because of their computational overhead higher level languages are not fast enough to allow real time analysis (Häder and Vogel 1990, 1991b). The direction of movement can be determined from relatively short track segments. Four images of the moving protists are recorded with a time gap of 80 ms between frames, digitized and stored in one of four memory quadrants each. Subsequently, the positions of all protists are determined in the first frame using the chain code algorithm described above. Starting from the centroids in the previous frame, the new positions of the cells are determined in the subsequent frames and stored in an array. The movement vectors are determined from the centroids in the first and final images. The deviation angle α from the stimulus direction can be calculated using

$$\alpha = \text{atn}\,\frac{y_2 - y_1}{x_2 - x_1} \qquad (8)$$

From these deviation angles direction histograms are constructed showing the preferred direction of movement of the population (Fig. 6a,b). The precision of movement of a population is determined using the Rayleigh test (Mardia 1972, Batschelet 1965, 1981) which yields an r-value between 0 (random orientation) and 1 (all organisms move precisely in the same direction).

$$r = \frac{\sqrt{(\Sigma \sin\alpha)^2 + (\Sigma \cos\alpha)^2}}{n} \qquad (9)$$

The lengths of the vectors can be used to determine the speed of movement of each organism using the time interval for the track segment read from the hardware clock of the computer. Velocity histograms can be calculated to show a correlation between the direction of movement and the mean velocity. E.g., *Euglena* cells have been found to move slower when swimming upwards than when swimming down (Fig. 7a, b) which is due to the passive sedimentation of the cells which superposes the active swimming. Movement under artificially increased accelerations was studied using

a slow rotating microscope centrifuge (NIZEMI, Häder et al. 1991a, b). The image of the swimming cells is recorded by a CCD camera during centrifugation and assayed by the image analysis system described above.

In order to analyze photophobic responses, e.g., in prokaryotic organisms the point of reversal or stop needs to be determined with respect to a step-up or step-down in light intensity (for definition see Diehn et al. 1977). For measurement of the photobehavioral responses in *Halobacterium halobium*, the cells are placed between a slide and a cover glass on the microscope stage (Takahashi 1991). Either dark field illumination or phase-contrast optics is used with a 32 - 40 x objective. Computer-linked video image analysis is used for tracking individual organisms (Takahashi and Kobatake 1982, Sundberg et al. 1985). The actinic light illuminates the cells vertically through the microscope optics. Either the time-lag between a light stimulus and the response or the fraction of the reversed cells within a given period after a light stimulus can be used as a measure of the magnitude of a response. Similar systems are used by Spudich (1991) and Marwan and Oesterhelt (1991). The joint effort of several groups has shown that step-down photophobic responses are mediated not by the proton and chloride pumping pigments in *Halobacterium*, bacteriorhodopsin and halorhodopsin, respectively (for review see Henderson et al. 1990, Khorana 1988, Lanyi 1990), but by a rhodopsin pigment called sensory rhodopsin I (Bogomoli and Spudich 1982, Spudich and Spudich 1982, Spudich and Bogomolni 1984). By transformation into a blue/UV absorbing form during its photocycle this pigment is also capable of mediating the step-up photophobic response. In addition, a second receptor, called phoborhodopsin or sensory rhodopsin II, was identified to mediate blue light step-up phobic responses (Takahashi et al. 1985a, Tomioka et al. 1986a, b, Spudich et al. 1986).

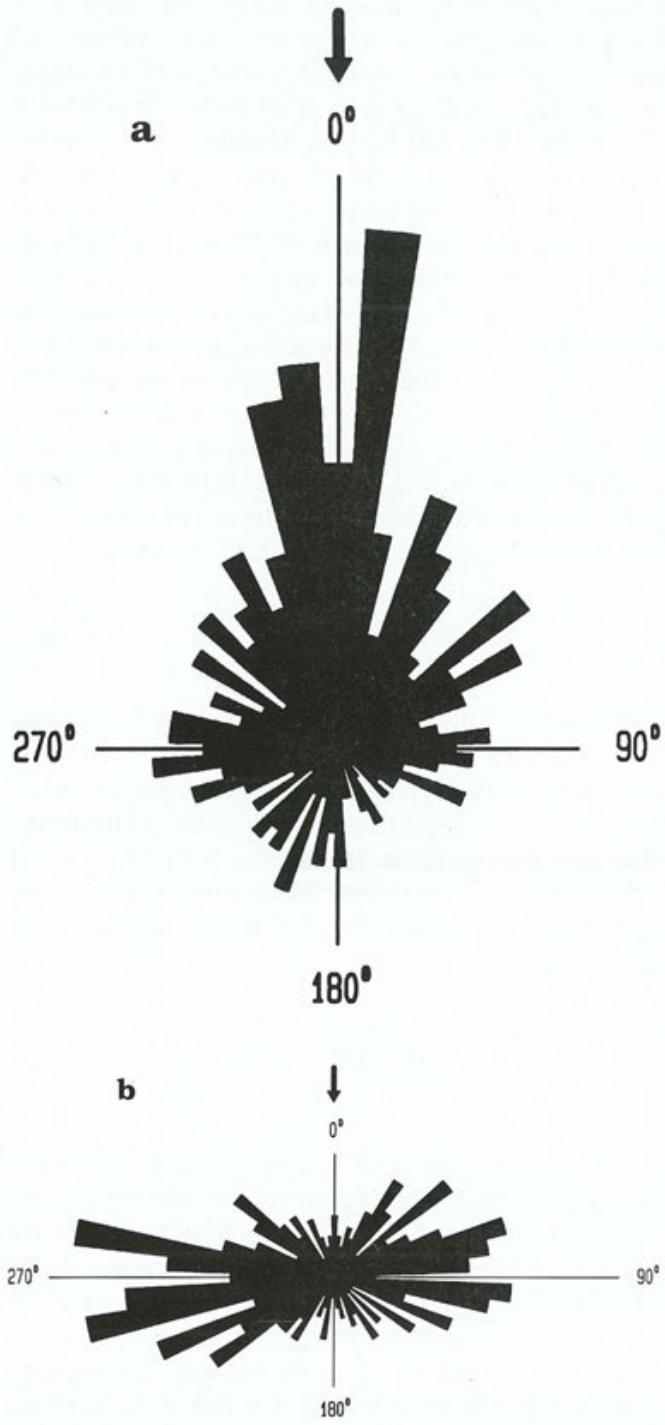

Fig. 6. Circular histograms of the dirction of movement of *Peridinium faeroense* in light impinging from 0° at (a, positive phototaxis) 555μ $Em^{-2}s^{-1}$ and (b, diaphototaxis) 6900μ $Em^{-2}s^{-1}$

Also in purple bacteria image analysis has been utilized successfully (Armitage 1991) to advance our knowledge beyond the stage of detailed microscopic observations accumulated over one hundred years (Engelmann 1882, 1883). In contrast to photokinetic effects, phobic responses seem not to be mediated by changes in the photosynthetic electron transport but rather by an intracellular metabolite which controls the frequency of flagellar switching. An electron transport dependent transduction protein might be involved which is also responsible for the integration with other sensory systems such as chemotaxis (Armitage and MacNab 1987, Armitage et al. 1990).

LASER LIGHT SCATTERING

While the movement tracks of photomotile protists can be followed by image analysis, some motion

parameters can only be measured by analyzing the light scattered from swimming microorganisms (Ascoli and Petracchi 1991). These techniqes have the advantage of being capable to detect fast responses not accessible to direct observation such as changes in the flagellar beating frequency (Ascoli et al. 1978a, b).

To measure velocities of cells or flagella, the Doppler shift of the scattered light is determined using coherent laser light and interferometric methods. Laser light can be regarded as a monochromatic plane wave, which undergoes a Doppler frequency shift when scattered by a moving particle. A direct detection method to measure the Doppler shift is heterodyne detection: the light scattered from the sample and part of the unperturbed beam fall on the photodetector and their superimposition is assayed spectrometrically. A good alignment of the scattered and heterodyning lights is required to warrant a useful signal to noise ratio; if wave surfaces are not parallel, interference fringes can arise on the photodetector.

Using a single *Euglena* cell in the measurement volume one obtains a sharp peak which oscillates to some degree around its mean position; this is because the cell axis oscillates during the motion which is generally true for other flagellated algae, which are greater than the wavelength of light and which move in a complex rototranslatory motion. Thus, the scattered light intensity is modulated both by the frequencies of the cell body rotation and by the flagellar beating. These frequencies appear in the heterodyne spectrum as lines around the Doppler line or can be detected directly, by measuring the power spectrum of the scattered light.

For flagellates such as *Haematococcus or Euglena* the photocurrent varies sinusoidally in time at two different frequencies: the higher at about 30 - 50 Hz represents the flagellar beating frequency and the lower at about 2 Hz the cell body rotation (Angelini et al. 1986). These techniques were used to study both phototaxis and photophobic responses in a number of flagellates (Angelini et al. 1986, Cantatore et al. 1989).

ELECTROPHYSIOLOGY

Earlier researchers have observed effects of externally applied electric fields in a number of protists including flagellates and ciliates (Verworn 1889, Jennings 1904). Since electric fields are no usual stimuli in the natural environment of protists this behavior has been interpreted as an indication that intracellular electrical potentials are involved in the sensory transduction of other external clues.

Murvanidze and Glagolev (1982) found that the direction of movement in the filamentous gliding cyanobacterium *Phormidium uncinatum* can be rever-

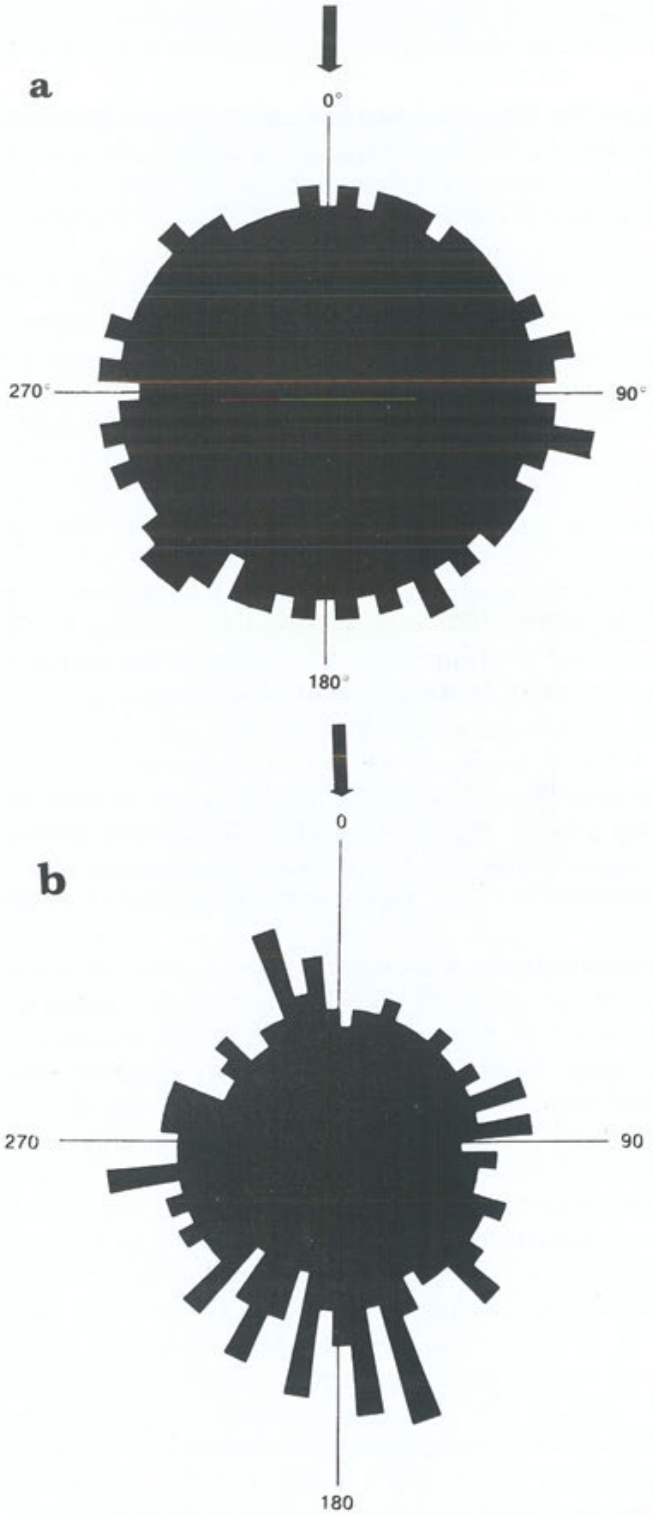

Fig. 7. Circular histograms of the velocity distribution of *Euglena gracilis* swimming in a vertical cuvette at (a) 1 g and (b) at an artificially increased acceleration of 4.5 g in the NIZEMI (Häder et al. 1991)

sed by the application and polarity reversal of an external dc field. Application of a moderate field also impaired photophobic responses in these organisms (Häder 1977). Light-induced electrical potential changes can be measured in the organisms using either extracellular or intracellular electrodes (Häder 1978a, b, 1979b). These electrical phenomena seem to be based on a series of events triggered by sudden changes in light intensity causing photophobic responses: in light a proton gradient is produced across the thylakoid membranes by a vectorial proton transport mediated by plastoquinone (Häder 1976, 1981, Häder and Poff 1982a). When a cell moves into a dark area this proton gradient breaks down causing a small potential change which in turn opens voltage-dependent, calcium specific channels in the cytoplasmic membrane (Murvanidze 1981, Murvanidze and Glagolev 1983, Häder 1982, Häder and Poff 1982b). The sudden massive influx of calcium ions further decreases the negative electric potential inside the cell (Häder 1986, 1987b). When an organism moves into a shaded area, this potential drop in the front cells of a trichome causes a reversal of the potential gradient along the length of the filament which controls the direction of movement in these organisms (Häder and Burkart 1982a, b).

Also some flagellates such as *Haematococcus* and *Chlamydomonas* utilize electrical potential changes in the sensory transduction chain of photoresponses (Sineshchekov and Litvin 1974, Sineshchekov 1991, Sineshchekov and Litvin 1982, Sineshchekov et al. 1990). In these small cells traditional intracellular microelectrode measurements gave no clear indication of the existence of specific photoelectric responses involved in photoreception due to the mechanical damage of the cell by microelectrode insertion. The first measurements of a blue light-induced electric response in flagellates were obtained by using extracellular microelectrodes placed closely adjacent to the cell surface (Sineshchekov et al. 1976). Lateron, the suction micropipette technique was used: the cell is sucked into the tip of a micropipette, causing two parts of the cell surface to become electrically insolated from each other. When a voltage is clamped between the inside and the outside of the pipette, an asymmetric photo-induced current can be measured directly (Sineshchekov 1988). An indication for the involvement of the measured light-induced electric potential changes in the phototaxis sensory transduction chain was given by the observation that potentials were only recorded when the membrane area overlaying the stigma, the putative photoreceptor organelle of the cells, was inside the suction pipette (Sineshchekov 1978, Ristori et al. 1981). Three potentials with different kinetics can be distinguished: a transient primary potential difference can be measured in the millisecond time range after the application of an intense flash. This is followed

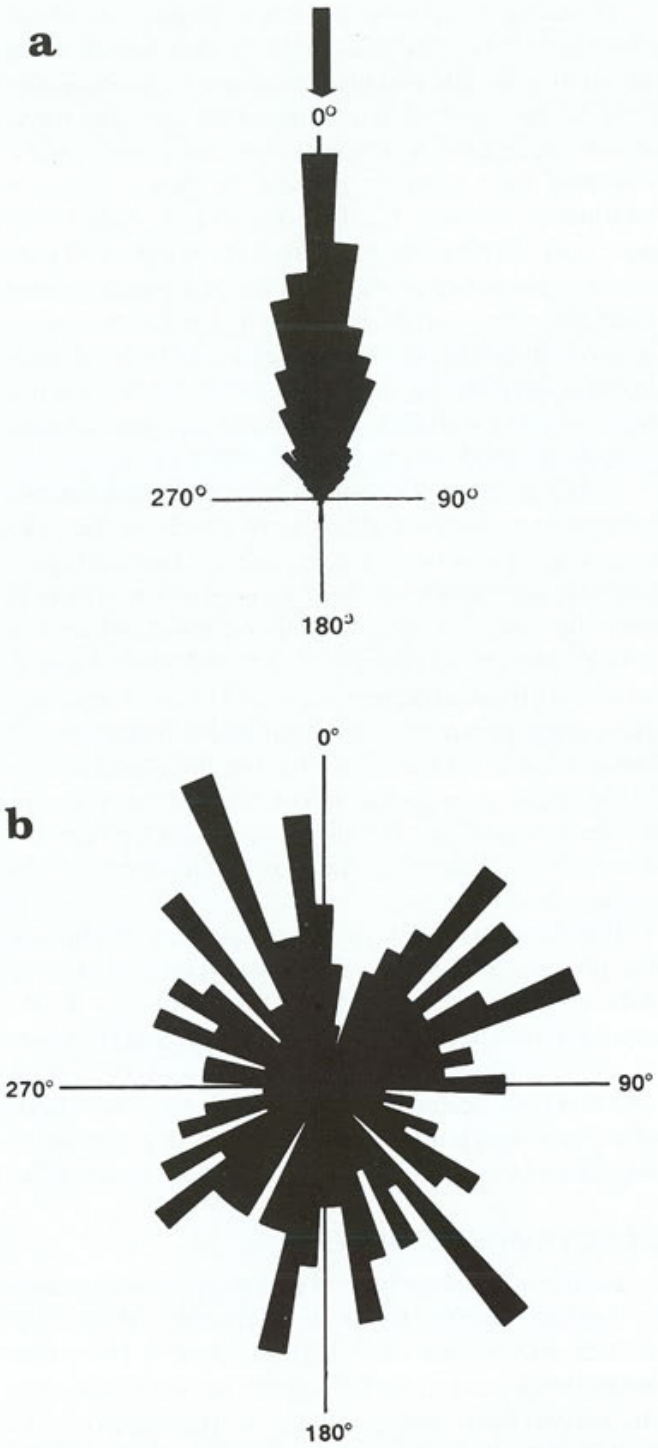

Fig. 8. Circular histograms of the direction of movement of *Euglena gracilis* swimming at (a) 1 g and (b) under microgravity (Häder et al. 1990)

by a permanent late potential with a lower amplitude and longer duration. The third event is an all-or-none regenerative response. The fast electrical signal is insensitive to variation of the ion composition of the medium which indicates that it precedes the biochemical steps of the light signal transduction and probably reflects the charge movement in pigment molecules (Sineshchekov 1991, Sineshchekov et al. 1990). Only short wavelength light elicits these responses which corresponds with the action spectrum for phototaxis in *Chlamydomomas* (Nultsch 1983).

SPACE BIOLOGY

Only fairly recently the advantages of space technology could be utilized to study the photobiology of protists. Phototaxis in many eukaryotic organisms follows a pronounced endogenous rhythm (Brinkmann 1976, Bruce and Pittendrigh 1956). In order to investigate whether this rhythm is induced or influenced by the earth's gravitational field, a *Chlamydomonas* suspension was flown during the D1 mission for close to two weeks in orbit using the US shuttle (Mergenhagen 1986). The degree of phototactic orientation was measured photometrically at regular time intervals. However, the results were not conclusive since the period of the rhythm under microgravity conditions was similar to the one of a culture kept in Hamburg, while the control population at the launching site in Florida had a distinctly different period (Mergenhagen and Mergenhagen 1987).

In addition to light, many flagellates and ciliates respond to the gravitational field of the earth and orient gravitactically (Brinkmann 1968, Bean 1984, Kessler 1985, 1986, Häder 1987a, Hemmersbach et al. 1991). In order to prove that the observed orientation is really elicited by the gravitational field of the earth and not by, e.g., chemical, thermal or magnetic gradients, which are known to induce behavioral responses in protists (Berg 1985, MacNab 1985, Mizuno et al. 1984, Poff 1985, Ofer et al. 1984, Frankel 1984, Esquivel and de Barros 1986), several space experiments were performed. The flagellate *Euglena gracilis* was used in one of these studies to test the effect of microgravity on orientation and motility (Häder et al. 1990, Häder and Vogel 1990). Not unexpected, the cells showed a pronounced random orientation even during the first minute of microgravity after launch of the ballistic rocket (Fig. 8b) while they showed a very precise gravitaxis under 1 g conditions (Fig. 8a). The swimming velocity was about 20% faster than in upward swimming cells at 1 g, which can be explained by the lacking Stoke's sedimentation under microgravity (see above). In another TEXUS experiment the effect of microgravity on the orientation and swimming velocity was studied in the ciliate, *Paramecium* (Hemmersbach-Krause, personal communication).

In future space experiments the phototactic orientation of flagellates is proposed to be investigated based on the idea that under terrestrial conditions phototaxis cannot be measured in the absence of gravity, which may influence the precision of orientation. Likewise, phototropism in fungi and higher plants is always superimposed by gravitropism at 1 g (Lipson and Terasaka 1981).

SPECTROSCOPIC ANALYSIS OF THE MOLECULAR PROPERTIES OF PHOTORECEPTOR PIGMENTS

The energetic events taking place on the molecular level during excitation and relaxation of a photoreceptor molecule have been subject of recent investigations. Traditional instrumentation has been used to determine the quantum yield of fluorescence, phosphorescence, photochemical reactions and other relaxation processes (see reviews by Lenci 1991, Song 1991). Recently, time-gated fluorescence spectroscopy using extremely short laser pulses in the picosecond time scale as an excitation source has found wide usage in photobiology and photochemistry (Cubeddu et al. 1991). This technique allows to discriminate between fluorophores with different fluorescence decay times by measurement in different time windows using time-correlated single photon counting. The decay time is very sensitive to the molecular environment of the photoreceptor and thus yields useful information on the photophysical properties of the chromophore. Using different wavelengths allows to measure time resolved spectra. This technique was employed to study the photochemical events during excitation of blepharismin, the proposed photoreceptor for photokinesis and photophobic responses (Matsuoka 1983a, b, Kraml and Marwan 1983, Scevoli et al. 1987) in the heterotrichous ciliate *Blepharisma* (Ghetti 1991). Linking the apparatus to a microscope allowed to perform measurements in single cells.

In ethanol two molecular species, emitting around 600 nm, with lifetimes 1 ns and 0.5 ns, respectively, were observed; in aqueous solution also two molecular species (lifetimes ca. 0.9 ns and 0.2 ns) were detected. Upon increasing the OH^- concentration, an anionic

species, emitting at 660 nm with a lifetime of 4-6 ns, appeared. By means of time-gated spectra, discriminating the contributions of species emitting with different decay times, it was possible to demonstrate that in aqueous solutions the formation of the anionic species takes place with a very low yield (Cubeddu et al. 1990). This means that the occurrence of deprotonation of blepharismin in its first excited singlet state as the primary molecular event in the photoreception in *Blepharisma* is still speculative.

An alternative hypothesis is the involvement of blepharismin-sensitized photodynamic reactions. Singlet oxygen might be produced also at low light intensities being the trigger signal for the photophobic response, similar to the proposed mechanism in *Anabaena variabilis* (Nultsch and Schuchart 1985). Crocetin, a partially water soluble carotenoid, that is an efficient quencher of singlet oxygen (Schuchart and Nultsch, 1984) gave no detectable reduction of the response in *Blepharisma* at fluence rates eliciting a phobic reaction in about 50% of the cells in control samples (Ghetti 1991), which excludes any significant contribution of photodynamically produced singlet oxygen in *Blepharisma* photoresponses.

Also recently the technique of laser flash photolysis has been used to study the primary photoprocesses of photoreceptor pigments in the reaction centers of photosynthetic protists (Navaratnam and Phillips 1991). Currently, pulsed solid state lasers, gas lasers or excimer lasers are used to generate pulses in the femtosecond range. The transient photoreceptor species generated by flash photolysis can be detected by optical absorption spectroscopy or optical emission spectroscopy. E.g., detection of excited singlet oxygen was based upon the latter method. Resonance Raman spectroscopy and photoacoustic spectroscopy were likewise used as detection mechanism (Nitsch et al. 1988). Figure 9 shows the experimental apparatus used by Navaratnam and Phillips (1991). This equipment can also be utilized to measure the extinction coefficient of triplet-triplet absorption and determine quantum yields.

BIOCHEMISTRY AND MOLECULAR BIOLOGY

The rigorous analysis of the molecular components involved in photoreception and sensory transduction

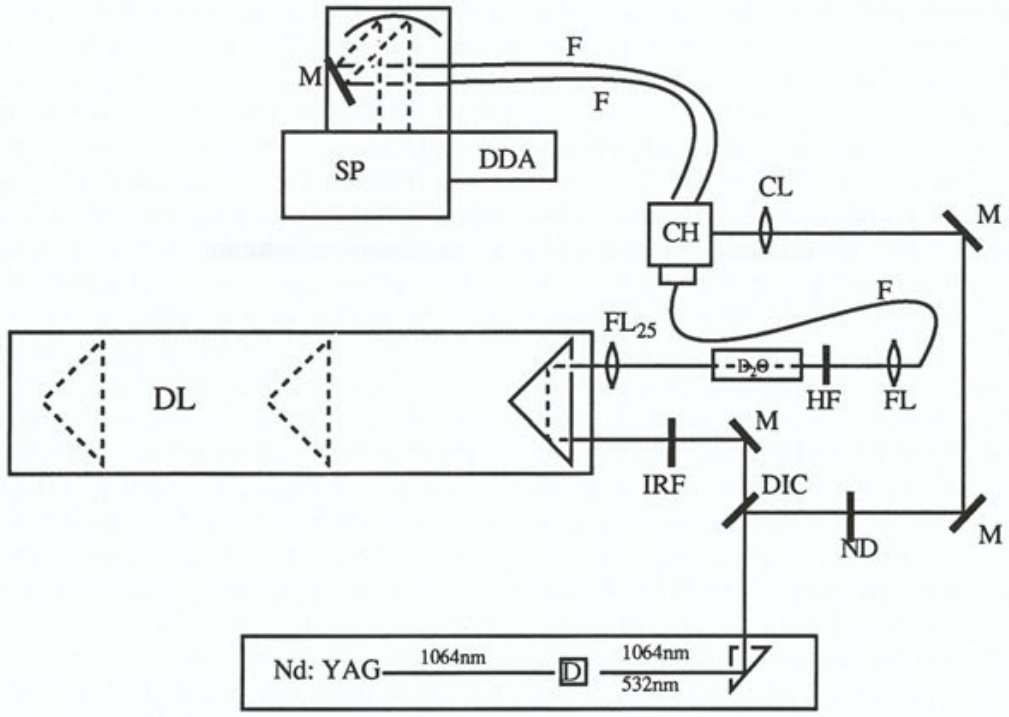

Fig. 9. Experimental apparatus for laser flash photolysis: D, frequency doubler; DDA, double diode array; DIC, dichroic mirror; DL, delay line; IRF, infrared pass filter; FL, focussing lens; H, heat filter; CL, cylindrical lens; M, mirror, F, fiber optics; CH, cell holder; SP, spectrograph; ND, neutral density filter (after Navaratnam and Phillips 1991)

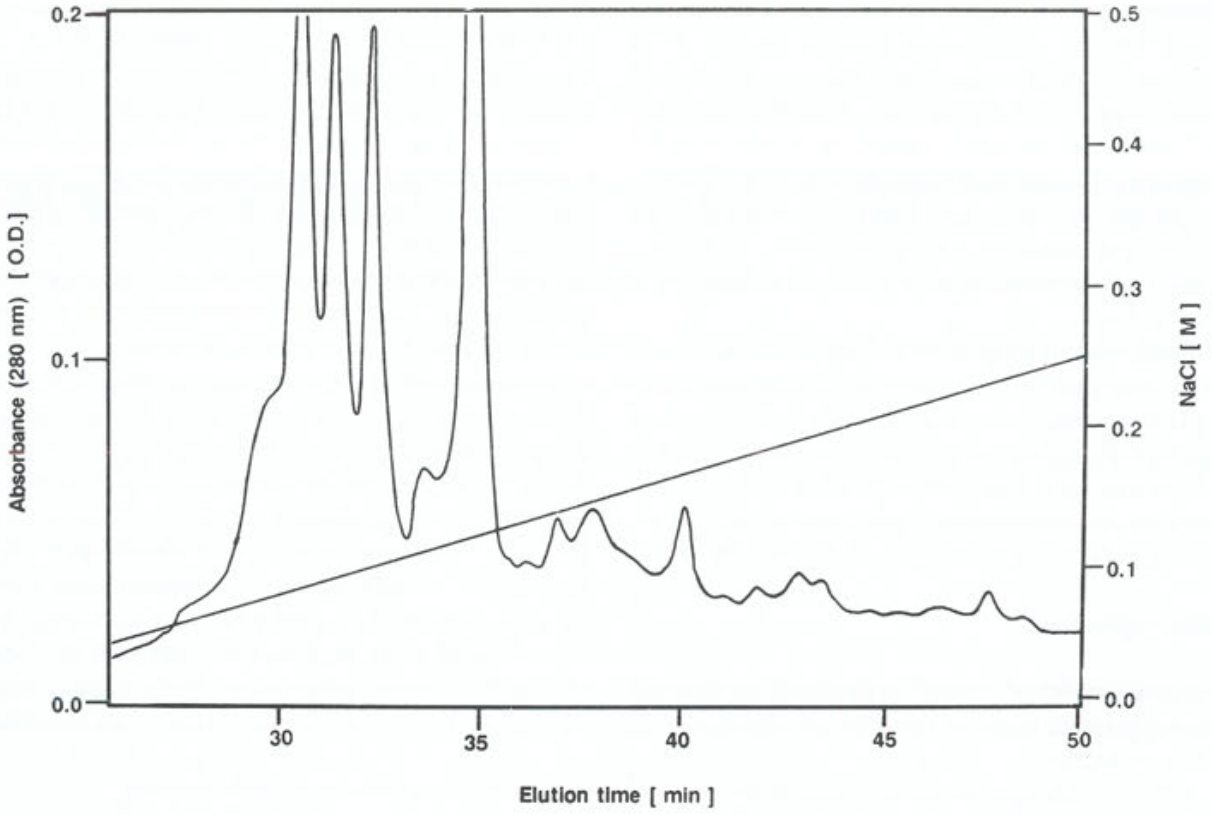

Fig.10. Elution diagram of proteins solubilized from the *Euglena* paraflagellar body running off a MonoQ ion exchange column (after Brodhun and Häder 1990)

of protists requires suitable tools to isolate and purify proteins, chromophoric groups, DNA and other biochemically important elements.

FPLC and ion exchange columns

One separation procedure relies on the differential retardation of substances by percolating them through a matrix of appropriate size beads or binding them to ligands on the matrix in a vertical column (Soderberg et al. 1983, Moreno-Lopez et al. 1981). Recent developments in this field are HPLC (high pressure liquid chromatography) (Tam et al. 1984, Burchiel et al. 1984, Welling et al. 1983) and FPLC (fast protein liquid chromatography) (Markey 1984, Johns et al. 1983, Karsnas et al. 1983) which differ from the simple column chromatography by using high or medium pressure, respectively, to force the substances to be separated through comparatively small columns. In addition to molecular sieve, affinitiy or reverse phase columns, ion exchange columns have proven to be useful. The separation priniciple is based on the fact that at a given pH solubilized proteins bind to positive or negative charges immobilized on the gel matrix in the column. Pumps of the FPLC produce a gradient of increasing NaCl concentration. The charges of the Na^+ and Cl^- ions compete with the proteins for the binding sites and at a given concentration remove the proteins which are separated based on their binding affinity.

The photoreceptor organelle responsible for phototaxis in the flagellate *Euglena gracilis* is supposed to be represented by the paraflagellar body (PFB), a swelling at the basis of the emerging flagellum (Benedetti and Checcucci 1975, Ghetti et al. 1985). The chromophore of the pigment has been suggested to be a flavin as indicated by action spectra for photophobic responses (Doughty and Diehn 1980), phototaxis and photoaccumulations (Diehn 1969) and microspectroscopic and fluorometric measurements. Gualtieri et al. (1986) improved a method recently described by Rosenbaum and Child (1967) to isolate the flagella of *Euglena* with

the PFBs attached (Gualtieri et al. 1989). Using such preparations it was shown that in addition to the presumed flavins, pterins are present in the PFB preparation (Galland et al. 1990). Fast protein liquid chromatography (FPLC) analysis demonstrated that the PFB contains four major protein fractions with the chromophoric groups still attached (Fig. 10). Fluorescence spectra of three separated fractions showed excitation and emission peaks indicative of pterins while the forth chromoprotein carried an additional flavin (Fig. 11).

Artificial or solar ultraviolet radiation affects motility and orientation in a number of marine and freshwater protists (Häder and Häder 1988a, b, 1989a, b, c). In *Euglena* the precision of orientation is impaired even after short exposure times. In parallel, FPLC analysis showed that the photoreceptor proteins are affected by ultraviolet radiation (Brodhun and Häder 1991).

Gel electrophoresis

A second group of protein separation techniques, gel electrophoresis, is one of the most powerful tools in modern biochemistry to purify molecules which differ in their physical and chemical properties (Lammel 1981, Rabilloud et al. 1985, 1986, Andrews 1986, Chrambach et al. 1989). The solubilized sample is applied to a gel matrix (e.g., agarose or polyacryl amide) and a dc voltage on the order of several hundred or thousand volts is applied across the gel by platinum electrodes to produce an electric field in which the individual proteins move electrophoretically because of their electric charge (native gel electrophoresis). Different size molecules are retarded to a different extent so that a separation is achieved.

Alternatively, the proteins are treated with sodium dodecyl sulfate (SDS), which binds to the side groups of the protein and has a negative charge. In the electric field the proteins move according to the charge they carry (SDS polyacrylamid gel electrophoresis = SDS PAGE). A different technique is based upon the fact that native proteins carry both positive and negative charges. Depending on the pH of the separation buffer one of the charges exceeds the other and at a specific pH both charges compensate each other, which is defined as the isoelectric point (IEP). This effect is used

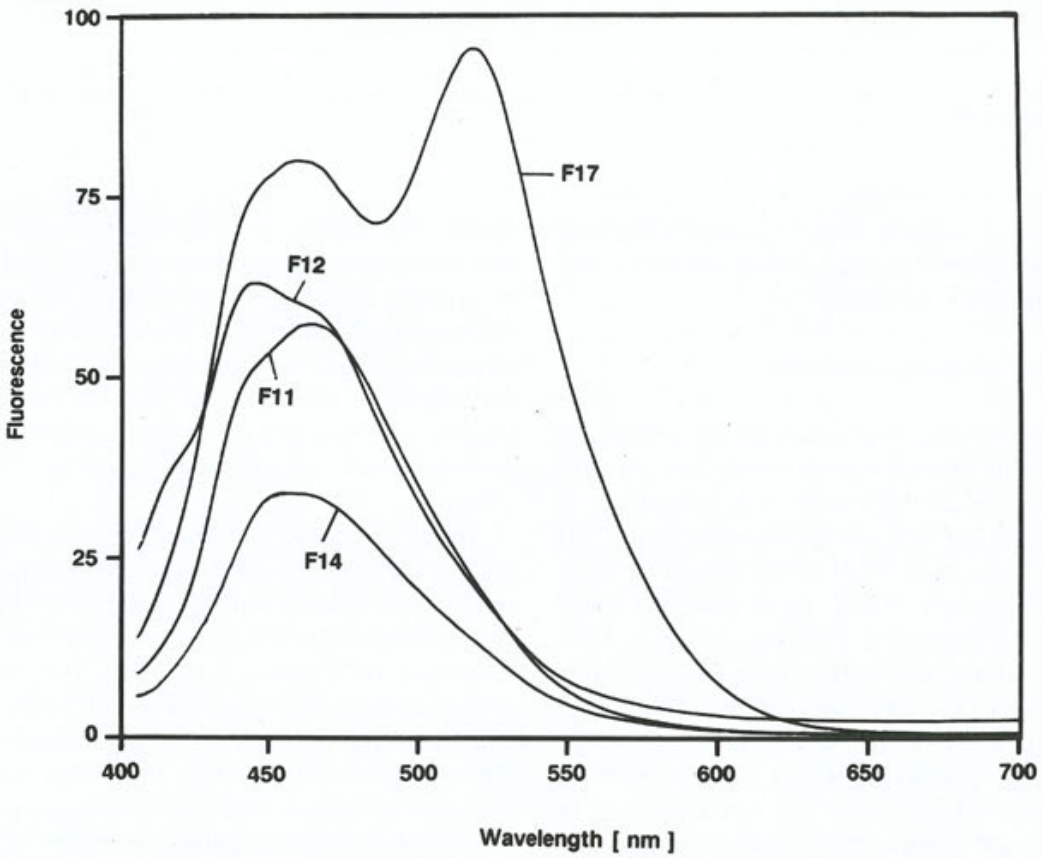

Fig.11. Fluorescence emission spectra of four FPLC MonoQ ion exchange column fractions excited at 380 nm indicating the presence of flavins and pterins in *Euglena* paraflagellar body (after Brodhun and Häder 1990)

by the technique of isoelectric focussing (IEF) (Patel et al. 1988, Righetti et al. 1986), where a pH gradient is established in the gel before the application of the sample and then the proteins are applied to the gel in which the proteins migrate in the gel until they move to a pH, which corresponds to its isoelectric point. Calibration proteins are added before the establishment of the pH gradient to indicate which region of the gel corresponds to which pH. Immunoelectrophoresis, affinity gel electrophoresis, agarose gel electrophoresis (Serwer 1980, Maniatis et al. 1982, McDonnel et al. 1977), chromatofocussing and other methods are also used for separation of the individual components in the protein mixture.

The proteins from the *Euglena* PFB separated by FPLC ion exchange chromatography were analyzed by gel electrophoresis: the pterin binding proteins have apparent molecular masses between 27 and 31.6 kDa and the flavin binding protein has an apparent molecular mass of 33.5 kDa (Fig. 12).

Especially for large numbers of proteins in the sample (Scheele 1975, Roggero and Pennazio 1990) two-dimensional (2-D) techniques are being employed for better resolution (Celis and Bravo 1984, Pahlic and Tyson 1985, Görg et al. 1987, 1988a, b, Strahler et al. 1987). Separation is a two step process where IEF or non-equilibrium pH gradient electrophoresis (NEPHGE) are used in the first direction (O'Farrell 1977a, b, Mattila et al. 1990) and usually SDS PAGE in the second direction perpendicular to the first (O'Farrell 1975, Tarroux 1983). Flat-bed gel electrophoresis (Dunn and Patel 1986, Görg et al. 1986) can be run horizontally or vertically and has a number of advantages such as an efficient cooling, small protein loss and high resolution during separation in thin (Dunn et al. 1984, Burghes et al. 1982) or ultrathin gels (Görg et al. 1980, 1982). These gels can be analyzed quantitatively using video image analysis (Häder and Truβ 1987). Recently, a computer program was developed to represent the gray scale differences in pseudocolor and in three dimensional graphics and also to quantify the amount of proteins in the individual spots (Häder and Kauer 1990, Häder 1991).

Molecular biology

Molecular biology is a fast growing field which can be employed for the study of photobiology of protists. One of the techniques to identify photoreceptor and other elements of a photosensory transduction chain is to produce mutants which can be done by chemical or radiative mutagenesis (Schneider et al. 1982, Williams 1978). The molecular biology approach is currently performed in *Dictyostelium discoideum* to identify the phototaxis photoreceptor gene(s) which is done by inserting a plasmid (pISAR) into the genome where it incorporates at random. The transformed strains are analyzed phenomenologically for phototactic defects. The plasmid and adjacent gene sequences are then extracted from behavioral mutants and the affected gene is cloned using the *E. coli* system and sequenced.

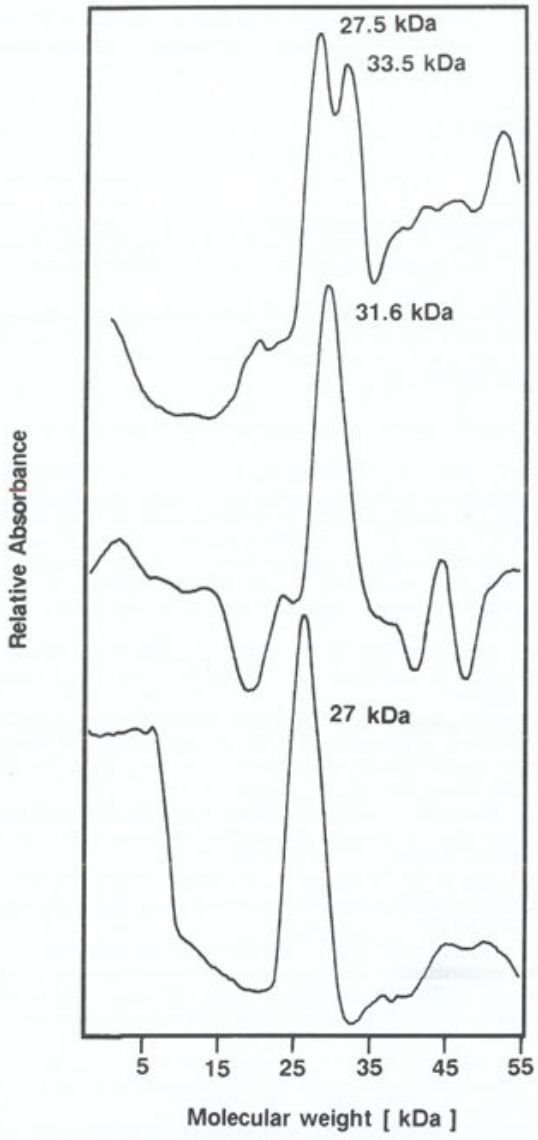

Fig.12. Gelscans of three FPLC fractions from *Euglena* paraflagellar body run on a Phastsystem SDS PAGE (after Brodhun and Häder 1990)

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ACTA PROTOZOOLOGICA

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Effect of Organic Calcium Channel Blockers on Ciliary Reversal in *Paramecium octaurelia* (strain 299 s)

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Summary. Short-lasting exposition of *Paramecium octaurelia* to calcium channel blockers (CCBs): nifedipine /N/, diltiazem /D/ and verapamil /V/ in concentration 1.7×10^{-5} M caused essential reduction of ciliary reversal /CR/ duration induced by 30 mM KCl due to partial blockade of calcium channels which were activated by potassium ions.

CCBs were applied in two series of experiments. In the first, blockers caused about 20% reduction of CR duration. In the second series of experiments ciliates were exposed to the following pairs of blockers: N + D, D + N; N + V, V + N; D + V, V + D. The succession of their addition were important only in the case of pairs : D + N and V + D , which showed about 40% decrease of CR.

In case of pairs N + V, V + N, N + D, D + V succession did not show influence on duration of CR. The achieved results demonstrate that drugs used, belonging to the three main classes of CCBs, inhibit the duration of CR of *Paramecium* in response to K^+ ions differently.

Key words. *Paramecium octaurelia*, calcium channel blockers, potassium chloride, ciliary reversal.

INTRODUCTION

The cell body of *Paramecium* is covered by thousands of cilia which beat in a coordinated fashion and propel the cell forward in left-handed spiral. *Paramecium* shows characteristic reactions in response to various kinds of external stimuli in form of long-lasting reversed beat of cilia or short-lasting "avoiding reaction" (Jennings 1906). Ciliary reversal /CR/ appears as a result of activation of calcium conductance within the ciliary membrane and the resulting influx of external calcium ions into the intraciliary space (for review see Dryl 1974).

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It is known that calcium ions enter the cell interior through calcium channels which are formed by a special class of integral membrane proteins (Saimi et al. 1987). Ehrlich et al. (1984) found that the ciliary membrane of *Paramecium* contains two types of channels. One of them is very selective for calcium and barium ions and strongly voltage-dependent, while the second one selects poorly among divalent cations and may be related to the mechanoreceptor of *Paramecium*. In addition to the voltage-operated calcium channel several other ion channels have been identified by various authors in electrophysiological studies of *Paramecium* (for review see Schultz et al. 1990). Like in other eukaryotic cells, the calcium current of *Paramecium* can be reduced by divalent cations (Wehner and Hildebrand 1985), but verapamil, D-600 or nifedipine have little effect (Kung and Saimi 1985). The organic substances like dihydropiridines (DHPs) and derivati-

ves of verapamil are effective in blocking the calcium currents of vertebrate heart and smooth muscle, but they are less effective in case of calcium channels in other membranes (Hagiwara and Byerly 1983).

Recent studies on blocking of calcium channels suggest the existence of different binding sites for nifedipine-like, verapamil-like and diltiazem-like compounds within calcium channel. Probably the binding site for DHPs is located close to the channel permeation machinery (selectivity filter) where the channel can be switched on or off (Glossman et al. 1982).

Results reported from several laboratories have presented evidence that diltiazem-like compounds stimulate DHPs binding, while verapamil-like compounds may inhibit DHPs binding. This may suggest the presence of separate binding sites for benzotiazepines (diltiazem derivatives) and for phenylalkylamines (derivatives of verapamil) as pointed out by Hosey and Lazdunski (1988). The last mentioned agents probably bind near the "inner mouth" of the calcium channel, since they affect the gating mechanism of the channel (Glossman et al. 1982).

The above mentioned differences in the localization of binding sites of various calcium channel blockers (CCBs) within channel suggest the possibility of interaction between blockers in dependence on the time of their application.

The aim of the present study is to analyze this problem in *Paramecium* which recently proved to be an adequate unicellular model for checking the behavioral effects of CCBs on eukaryotic cells (Ucieklak and Dryl 1990). The hypothetical structure of the proteinaceous calcium channel within the cell membrane is shown in a schematic picture, Fig.1 (adapted from Schramm and Towart 1985).

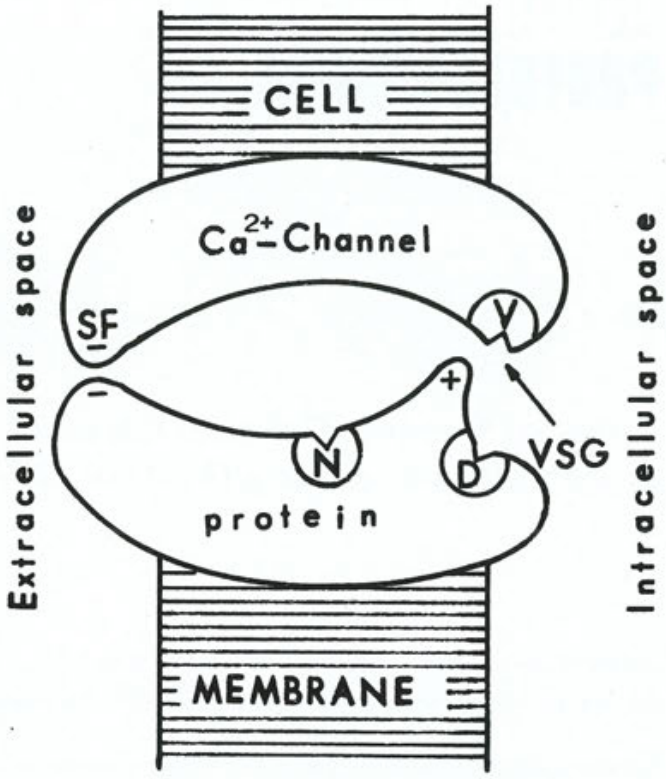

Fig. 1. Hypothetical schematic presentation of the voltage sensitive calcium channel within the cell membrane. SF - "Selectivity Filter". It posses negative charges. At this site the inorganic calcium antagonists can block the calcium channel. VSG - "Voltage Sensitive Gate". Its function can be modulated by organic calcium channel blockers, which have different binding sites inside the Ca^{2+} channel. N, V, D: proposed binding sites for Nifedipine, Verapamil and Diltiazem (The scheme was adapted from a review article by Schramm and Towart 1985)

MATERIAL AND METHODS

Experiments were carried out on *Paramecium octaurelia* strain 299s, cultivated in axenic medium (Soldo et al. 1966, 1969) at 22-24°C. Before starting experiments the ciliates were harvested by means of negative geotaxis, washed twice in buffer solution 1mM $CaCl_2$ + 1 mM Tris/HCl (pH 7.2) and left for starvation during a period of 20 h (Ucieklak and Dryl 1990). Potassium chloride and calcium channel antagonists were also prepared on the basis of the above mentioned solution.

Experimental solution in double concentration was added to the ciliates in buffer solution in depression slide. After appropriate time (depending on experiment) double concentration of KCl with appropriate CCB was added and time of ciliary reversal was estimated. Duration of ciliary reversal of 50% of 50 specimens of paramecia, by means of stopwatch, under the low magnification microscope (10x), was measured. Calculations were done on the basis of 10 repeated series of experiments.

The first series of experiments was carried out on paramecia which were preincubated in 1.7×10^{-5} M solution of calcium channel blocking (CCB) substances (nifedipine, verapamil and diltiazem) for three minutes and afterwards the 30 mM KCl solution was added. Experimental solution contained CCBs in concentration used as above.

The second series of experiments were performed on ciliates exposed to the above mentioned concentration of the first CCB substance for the first 90 seconds, and then to a mixture of the first and second CCB solutions for the following 90 seconds. Then the 30 mM KCl solution containing the same concentration of CCBs was added. The following combination of CCBs were investigated by the above mentioned procedure: D + (N + D), N + (D + N), N + (V + + N), V + (N + V), D + (V + D) and V +(D + V). Mean time of ciliary reversal evoked by the 30 mM KCl in buffer solution was the control. Experimental data in Fig. 2 have been expressed in percentage in relation to the mean value of the control.

RESULTS AND DISCUSSION

The data from the first series of experiments showed that the single CCBs (N, D and V) caused about 20%

Table 1

Duration of CR of *Paramecium octaurelia* (strain 299s) evoked with 30 mM KCl in the presence of CCBs. N – Nifedipine, D – Diltiazem, V – Verapamil

Applied Ca^{2+} channel blockers	Duration of K^+ – induced CR in seconds mean values, SD
D	56.2 ± 2.1
N	50.5 ± 1.4
V	54.5 ± 2.8
D + (N + D)	41.5 ± 2.1
D + (V + D)	45.3 ± 1.8
N + (D + N)	49.6 ± 3.0
N + (V + N)	45.0 ± 2.9
V + (N + V)	44.3 ± 2.6
V + (D + V)	39.8 ± 1.0
Buffer solution	69.2 ± 1.1

decrease of CR duration, induced by 30 mM KCl solution in relation to the control. This was in good agreement with data reported previously by the authors (Ucieklak and Dryl 1990).

In the second series of experiments the succession of CCBs application proved to be important only in the case of pairs: D + N, N + D and D + V, V + D. It is evident from the data included in Table 1 and Fig. 2 that the blocking effect of D + N was stronger than N + D and the action of V + D was much stronger than that of D + V. On the other hand, the successive application of CCBs in case of N + V and V + N showed no influence on the induced duration of CR by potassium chloride. The strongest effect was observed in case of pairs: D + N and V + D, which showed about 40% decrease of the CR time in relation to control.

This is in accordance with the observations performed on *Stylonychia mytilus* and *Fabrea salina* (Dryl and Totwen-Nowakowska 1985, Dryl and Łopatowska 1990): in both cases organic CCBs caused shortening of potassium-induced ciliary reversal. However, these effects were less pronounced than those observed with divalent cations.

Experiments with isolated flagella of *Chlamydomonas reinhardtii* revealed that specific binding sites for diltiazem, verapamil and nifedipine occur in plasmic and flagellar membranes (Dolle and Nultsch 1988). Diltiazem binding is inhibited by verapamil. Ligand displacement experiments with isolated flagellar membranes using organic CCBs, diltiazem, verapamil and 1,4-dihydropyridine, nimodipine gives the first evidence that for each drug a specific binding site exists with about the same affinity as established for the plasma

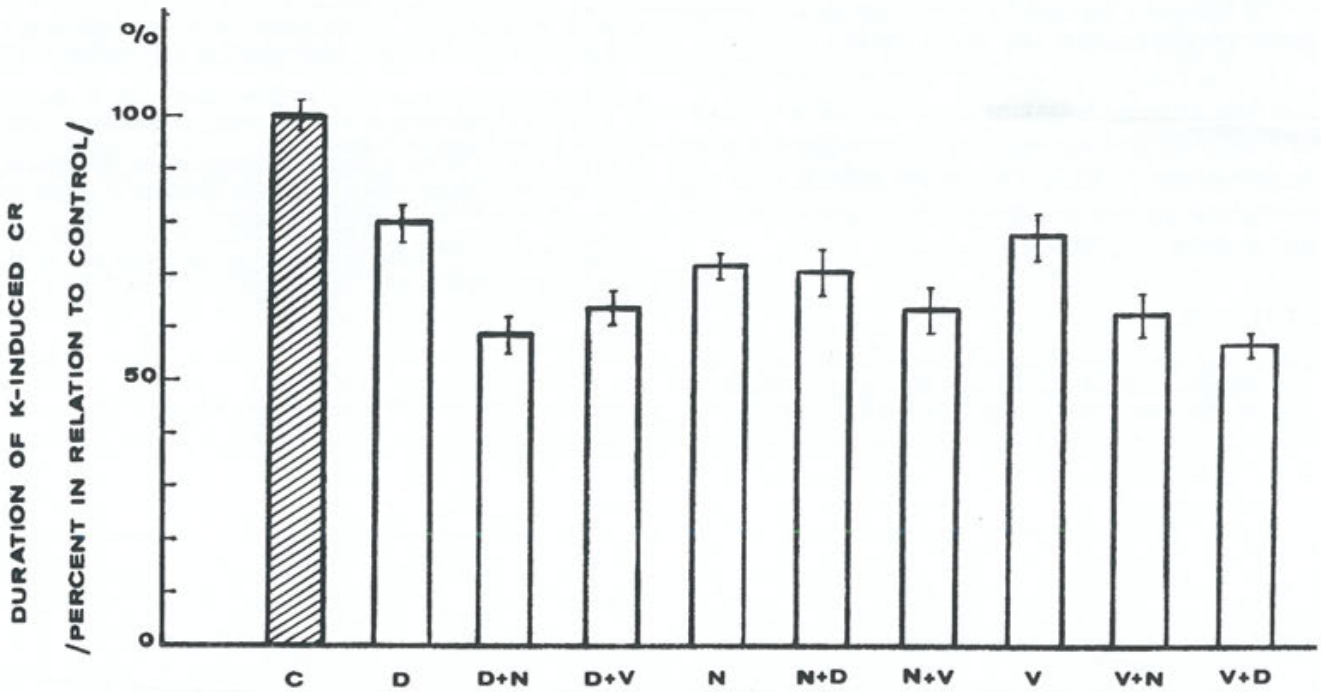

Fig. 2. Duration of ciliary reversal (CR) evoked with 30 mM KCl in the presence of calcium channel blockers of *Paramecium octaurelia* (strain 299s). Duration of CR of paramecia exposed to 30 mM KCl solution devoid of CCBs was the control. N - Nifedipine, D - Diltiazem, V - Verapamil

membrane fraction. It is tempting to speculate that the same situation may exist in *Paramecium*: the stronger action of the D + N then V + D seems to support this supposition.

Moreover, the similar effect was observed in guinea pig skeletal muscle T-tubules (Striessnig et al. 1986). Striessnig et al. (1986) brought evidence that purified calcium channels have three allosterically coupled drug receptors. They found that diltiazem markedly stimulates 1,4-dihydropyridines binding to the isolated calcium channel. It was interesting that verapamil binding was stimulated by antagonistic DHPs whereas the agonistic ones (for example BAY k8644) were inhibitory.

Some similarities between Ca^{2+} channels in protozoan and metazoan cells have been recently discussed. The *Paramecium* calcium channel has many features in common with one class of Ca^{2+} channels in vertebrate tissuses called "T" channel (Ehrlich et al. 1988).

Another hypothesis has been put forward that the Ca^{2+} channels of lower organisms resemble the Na^{+} channel in higher organisms. It is interesting that the Na^{+} channel activator veratridine activates the Ca^{2+} channel of *Paramecium* (Schultz et al. 1990). The biochemical composition of the Ca^{2+} channel of *Paramecium* is still unknown. As concerns the inhibition of the Ca^{2+} current in voltage clamp studies, the anticalmodulin agent W-7 was found to be the most potent, however, the mechanism of its action is not known yet (Hennessey and Kung 1984).

The observations achieved in this study suggest that three well-known CCBs inhibit the duration of backward swimming of *Paramecium* in response to higher concentrations of KCl. The results suggest also the possible competitive action of CCBs in case of applying them together in different succession.

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ACTA
PROTOZOOLOGICA

Studies on Biochemical Indicators of Virulence in the Amoebae of the *Acanthamoeba* sp.

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Summary. The degree of virulence of amoebae is most often defined by tests on mice. Biochemical investigations to differentiate between invasive and non-invasive strains of *Acanthamoeba* sp. are scarce. The aim of our study was to demonstrate a possible correlation between some biechemical indicators and the degree of virulence of investigated strains. The results testify to a distinct correlation between the activity of trypsin and peroxidase and the degree of virulence of the strains. The strains that kill the greatest number of infected animals are at the same time characterized by the highest value of the activity of both the enzymes.

Key words: *Acanthamoeba* sp., peroxidase, trypsin, virulence

INTRODUCTION

Amphizoic amoebae of the *Acanthamoeba* genus have been studied by protozoologists and parasitologists for the last twenty years. These ubiquitous amoebae may constitute an ethiological factor of granulomatous amoebic encephalitis (GAE) and other diseases in humans and animals (Gullet et al. 1979, Borochowitz et al. 1981, Visvesvara et al. 1983, Moore 1988, Wiena et al. 1988).

Pathogenic species of amoeba isolated from clinical cases in human or from natural environment as a potential source of invasion constitute the major objects of investigations. The degree of invasiveness as well as that of virulence of the amoeba are most often defined by tests on mice. Such biological evaluation of the strains is a long process as it may take over a month for the infected animals to die and because it requires taking into account such factors as the quantity of the inoculum, period of generating the amoebae, temperature of incubation as well as kinds of strains of mice used in the experiment and their age. Because of the above mentioned reasons, last years saw a number of publications whose goal was to identify biochemical indicators of pathogenicity in this group of amoebae (Hadaś 1982, Kasprzak et al. 1986). However, only a few studies on the enzymatic differences between pathogenic and non-pathogenic strains of *Acanthamoeba* exist and the same is true of the studies concerning strains of different degrees of virulence. Yet it seems that these biochemical investigations may prove useful in differentiating these strains. Our study was aimed at eventually demonstrating the existence of possible correlation between the degree of virulence and some biochemical indicators of the amoebae of *Acanthamoeba* species.

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MATERIAL AND METHODS

The following strains of *Acanthamoeba castellanii* were used: (a) strains: 2094 P, 2114 O, 2202 P, 2217 M, 2217 P, 2217 O isolated from different organs (M from brain, P from lung and O from eye) of mice infected with populations of amoebae from environment (Mazur et al., in press), (b) strains: 2370 M, 2382 M isolated from brain of mice infected with amoebae isolated from water habitat (Mazur and Sawicka, in press), (c) standard strains of *Acanthamoeba castellanii*: 309 - isolated by Kasprzak and Mazur (1972), characterized by a pronounced loss of virulence through long-term cultivation in vitro (Kasprzak et al. 986); Neff - non-invasive for mice in intranasal method of inoculation (Neff 1957).

The amoebae from samples of water and mud as well as from samples taken from infected tissue of animals were cultivated on agar medium (NNE) prepared according to Culbertson et al. (1965). For biochemical examination the amoeba were cultivated in axenic cultures on liquid medium BC + hs (Bacto-Casitone and horse serum) according to Cerva (1969).

The isalation of the amoeba, their cloning and multiplication as well as establishing and conducting cultures on liquid medium and taking samples from infected animals were conducted according to the procedures referred to earlier (Kasprzak and Mazur 1973) .

Specific identifications of the amoebae were conducted according to morphological criteria (Page 1976) and by electrophoretic examination of the protein of the amoebae (Hadaś et al. 1977).

The amoebae from liquid 4-day old cultures were centrifuged at 900xg for 20 minutes and then rinsed with distilled water. The sediment was suspended in extraction buffer (0.1M potassium phosphate, pH 7.0) at the ratio of 1:4 v/v and homogenized at 4°C. The homogenates were centrifuged at 15000xg for 30 minutes. Obtained supernatants were stored in ice untill the moment of determination.

The content of the protein in the supernatants was determined by the method of Lowry et al. (1951).

The enzymatic activity of peroxidase in supernatants from the amoeba was determined spectrophotometrically (Putter 1974).

The enzymatic activity of trypsin was determined colorymetrically using Azocoll (Calbiochem) as a substrate.

RESULTS

The results are presented in Table 1. High activities of peroxidase (over 0.200 IU/mg of protein) and of trypsin (11.20 - 28.49 IU/mg of protein) were found in the strains isolated from the brains of the infected mice. The activity of peroxidase of the strains isolated from lungs and eyes was low as a rule (0.204 -and less - IU/mg of protein), with equally low values of the activity of trypsin (5.74 - 15.32 IU/mg of protein). Strain 2202 P was an exception. Decidedly low activity of both examined enzymes was found in the non-invasive strain Neff *(Acanthamoeba castellanii)*: 0.117 IU/mg of protein for peroxidase and 0.12 - 0.20 IU/mg of protein for trypsin.

The degree of virulence expressed in the ratio of the number of animals infected to the number of animals destroyed was highest in the strains isolated from brain and was to a large extent concurrent with the activity of both examined enzymes. Whenever the activity of peroxidase was higher than 200 IU/mg protein, about 50 per cent of infected animals yielded to the invasion and, in most cases, died.

The enzymatic activity of peroxidase of strain 309 was slighty increased after 5 passages while the activity of pepsin was increased more than twice.

DISCUSSION

The virulence of strains of various pathogenic species of *Acanthamoeba* is usually examined by biological studies on animals or in tissue cultures. Mazur and Sawicka (in press) reviewed methods that evaluate biological characteristics of amoebae originally living in the environment. Among other issues, their study emphasises the importance of the technique of zymograms to define enzymatic proteins. Studies aimed at designating the biological identity within one species (e.g. degree of virulence) have been conducted for quite a long time. Sargeaunt and Williams (1978) proved that by examining the activity of some enzymes, differences between various strains of *Entamoeba histolytica* displaying different degrees of virulence can be identified. Such studies are scarce, however.

Our enzymatic study points out to a certain regularity, that is: higher activity of peroxidase and trypsin in strains of *Acanthamoeba castellanii* isolated from the brain of infected animals than in those isolated from other tissues. It is also to a high extent concurrent with the degree of virulence of these strains. It may be presumed that the colonization and proliferation of the amoebae influence their malignancy in the brain tissue to a degree higher than in other tissues.

We can also notice some evident correlation between the degree of virulence and the activity of both the examined enzymes in particular reisolates (strain 309 *A. castellanii*). The virulence of the strain is convergent with the high activity of trypsin . Non-invasive strain Neff *(A. castellanii)* is characterized by the lowest activity of the enzymes and this is particularly clearly seen in the case of trypsin.

Peroxidases are anti-oxidant enzymes that protect organisms against toxic activity of peroxides which are produced in the process of respiration and in the process of phagocytosis. Their major role is to protect the parasites from being eliminated from the host organism. So far, the presence of peroxidase has been demonstrated in *Leishmania tropica, L donovani,*

Toxoplasma gondii, Plasmodium berghei (Callahan et al. 1988) and in *Acanthamoeba* sp, (Hadaś 1991). As far as trypsin is concerned, this lyzosomal enzyme is believed to be responsible for the pathogenicity and penetration of *Entamoeba histolytica* to host tissues

Table 1

Virulence and specific activity of peroxidase and trypsin in different strains of *Acanthamoeba castellanii*. Enzymatic activity is expressed in IU/mg of protein (mean value ± SD, n = 5–7)

Strain	Virulence*	Peroxidase	Trypsin
2202 P	4/3/2	0.252 ± 0.015	16.06 ± 1.64
2217 M	n.e.	0.277 ± 0.023	28.49 ± 3.45
2217 P	n.e.	0.115 ± 0.014	15.32 ± 3.23
2217 O	12/3/3	0.116 ± 0.011	5.74 ± 1.56
2219 P	5/2/2	0.204 ± 0.012	8.53 ± 1.17
2382 M	7/7/7	0.200 ± 0.014	12.80 ± 2.45
2370 M	7/7/5	0.200 ± 0.020	11.20 ± 1.90
2114 O	10/4/2	0.183 ± 0.021	8.09 ± 1.32
2094 P	10/4/2	0.167 ± 0.009	14.04 ± 2.52
309 **	15/n.e./4	0.153 ± 0.025	5.70 ± 0.92
309 ***	6/4/2	0.161 ± 0.012	12.30 ± 2.15
309 ****	n.e.	n.e.	8.80 ± 1.10
Neff	5/0/0	0.117 ± 0.021	0.12 ± 0.11
Neff ****	5/0/0	n.e.	0.20 ± 0.20

* expressed as the relation of the number of animals inoculated to infected to dead, ** strain isolated from the cysts stored in 4°C, *** strain freshly passaged by mice, **** earlier data (Hadaś 1982), n.e. – not examined

(Meerovitch 1977). It is possible that in free-living amoebae it either plays a similar role or, else, it testifies to the pathogenicity of the species (Hadaś 1982).

Hitherto obtained data (among others: De Jonckhereere 1982, Kasprzak et al. 1986, Yong-Kyu et al. 1988) as well as our observations testify to the fact that the value of the activity of some enzymes, peroxidase and trypsin included, may come to be totally reliable biochemical indicators of the degree of virulence of the amoebae of *Acanthamoeba* sp. It may become possible to establish the boundary values of the biochemical indicators of pathogenicity, below or above which a strain is or is not invasive for animals in intranasal infection. It will however require further, more extensive investigations.

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ACTA
PROTOZOOLOGICA

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Prostaglandins of *Tetrahymena pyriformis* GL-C and *T. rostrata*

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Summary. The investigation was aimed at isolating and identifying prostaglandins from two species of *Tetrahymena: T. pyriformis* GL-C, a free-living, non-parasitic form, and *T. rostrata*, a parasitic form. Separation of prostaglandins was made by thin-layer chromatography on a silica gel. One kind of prostaglandin, PGE_2, was identified. Moreover, the detection and activity of a prostaglandin synthetizing enzyme system was studied using cytochemieal methods. A higher activity of prostaglandin synthetase was found in *T. rostrata*.

Key words: Ciliates, *Tetrahymena pyriformis* GL-C, *T. rostrata*, prostaglandins

INTRODUCTION

Prostaglandins (eicosanoids, PGs) are widely distributed and play important roles in almost all mammalian tissues and fluids (Horrobin 1978). They are often mentioned in the context of mammalian physiology and pathophysiology. These compounds are importan in human health and disease (Zaorska 1986). Mammalian tissues synthesize up to 30 different kinds eicosanoids, each with a different biological activity. Even a single organ can synthesize different forms of eicosanoids which may have a similar or opposite biological activity in that organ. For example, prostaglandins and leucotriens play significant roles in the mammalian cardiovascular system. The PGE_2 and PGI_2 series are usually vasodilator in most vascular beds, whereas $PGF_{2\alpha}$ and TX_2 may act as vasoconstrictor. The local formation of PGs may be necessary for the regulation of blood flow within particular organs of the body. These compounds affect essentially all aspects of female mammalian reproductive functions from the release of gonadotropic hormones from the pituitary gland to parturition and lactation (Mustafa and Srivastava 1989).

Until the first report on prostaglandins appeared in an invertebrate animal (Weinheimer and Spraggins 1969), the PGs were not studied from non-mammalian organisms. By now it is kwown that PGs are present in many groups of organisms. They occur in invertebrates (Horrobin 1978, Grzywacz and Szkudliński 1984, Salafsky et al. 1984) and several vertebrate species (Srivastava and Mustafa 1984, Mustafa and Srivastava 1989). Prostaglandins were also found in some protozoa. Das and Padma (1977) have reported PGs from *Entamoeba histolytica* and Hadaś (1987, 1988) from some species and strains of *Acanthamoeba*.

Unsaturated fatty acids, especially linoleic and arachidonic acid serve as precursors for prostaglandins. It is interesting to note that studies in lower animals have likewise shown the occurrence of these

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specific fatty acids. In *Tetrahymena* there are several unsaturated fatty acids (Erwin and Bloch 1963, Hill 1972) which may exert their biological effect after having been converted into prostaglandins. Florin-Christensen et al. (1986) found a phospholipase in *Tetrahymena*; it was an enzyme necessary in the biosynthesis of PGs, and shown to be of type A1 (E.C. 3.1.1.32). These findings suggest that prostaglandins can be present in *Tetrahymena*.

Quantitative differences between prostaglandins from pathogenic and non-pathogenic strains of *Acanthamoeba* (Hadaś 1987) suggested that these compounds may play important roles in pathogenicity of parasites.

The present study has been directed towards cytochemical and biochemical examination the presence of prostaglandin synthetase and prostaglandins in parasitic and free-living species of *Tetrahymena*.

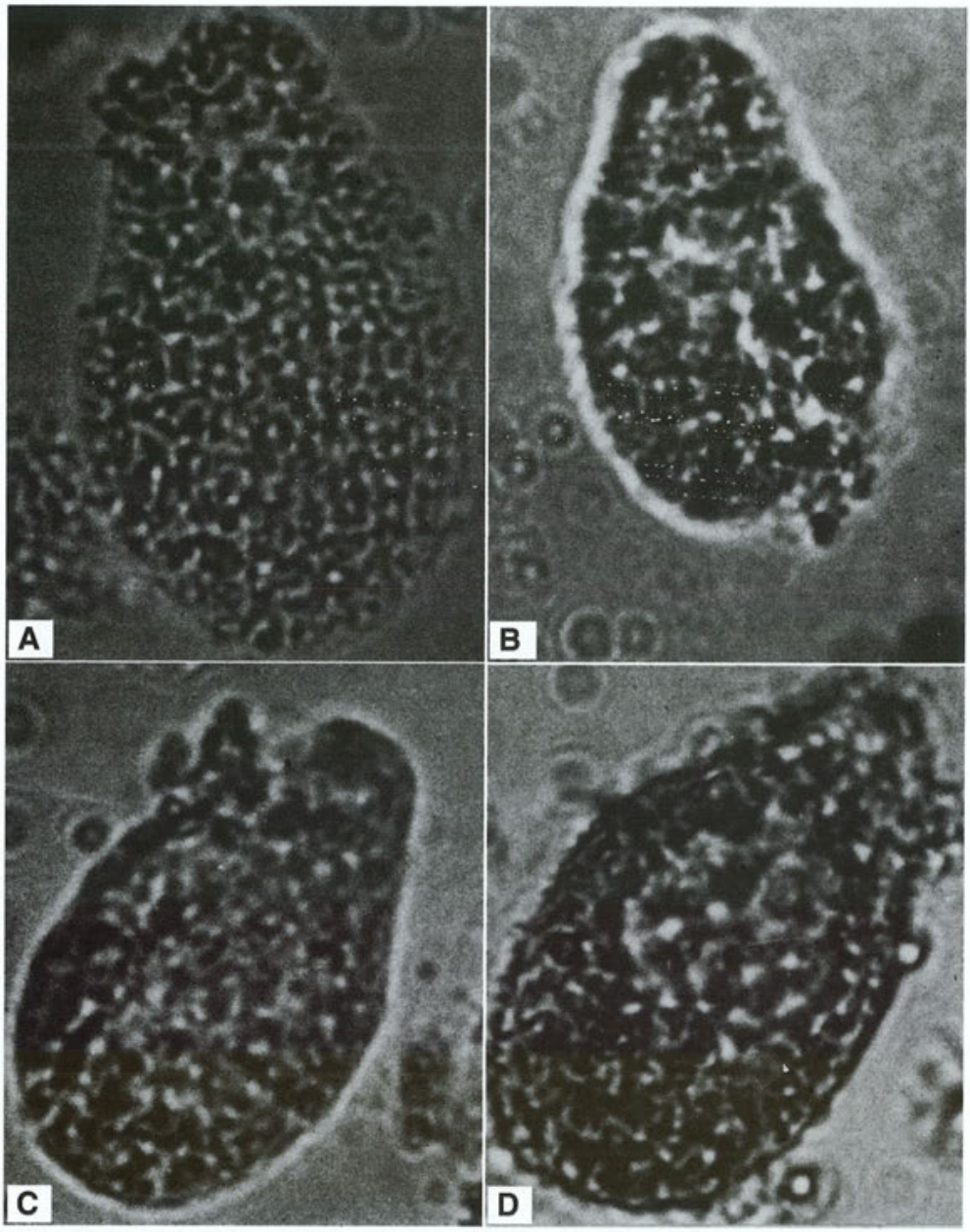

Fig. 1. *Tetrahymena pyriformis* GL-C (A – control, B – investigated cell) and *T. rostrata* (C – control, D – investigated cell) after incubation on prostaglandin synthetase activity with DAB, KCN and with or without (control) arachidonic acid. (x 250)

MATERIAL AND METHODS

The cells

The material used in the study were the ciliates *Tetrahymena pyriformis* GL-C and *T. rostrata*. *T. pyriformis* GL-C is an amicronucleate strain derived from a free-living form and *T. rostrata* is a parasitic form, isolated from the renal organ of *Zonitoides nitidus* (Mollusca, Gastropoda) and the cells were cultivated in the laboratory during 8 years. The free-living and parasitic species were cultivated in Erlenmayer flasks containing 50 ml of medium (1.5% proteose-peptone + 0.1% yeast extract + salts) (Plesner et al. 1964) at 25°C. The cells used in the study were at a stationary phase of growth (about 144 h after inoculation). The concentration of potential precursors of PGs at this phase is higher than at other phases of growth (Erwin and Bloch 1963).

Localization of the prostaglandin synthetase in cells

The localization of the prostaglandin synthetase was made according to the method of Ledwozyw et al. (1986) and modified by Hadaś. The cells were washed several times with the buffer and then frozen (-25°C). Fixation is not absolutely necessary because a loss in sensitivity of about 50% was noted during staining (Janszen and Nugteren 1971). Histochemical reaction on the prostaglandin synthetase was performed by incubation the cells in a staining mixture containing 100 μm arachidonic acid, 1 mM 3,3'-diaminobenzidine, 2 mM potassium cyanide in 100 mM Tris-HCl buffer pH 8.2. The reaction was stopped by rinsing the cells with water and then with cacodylic buffer. The samples were embedded in 2% gelatin in 50% glycerin. The controls were stained by a mixture without arachidonic acid.

Extraction of prostaglandins and chromatography

Prostaglandins were extracted according to the method of Gréen et al. (1978) described in details by Hadaś (1987). Separation of prostaglandins was made by thin-layer chromatography on silica gel (Silica Gel 60 F-254 - Merck). Prior to use, the plates were activated at 110-115°C for 30 min. The following solvent systems were used, ethyl acetate: acetone: acetic acid - 80:10:10 and ethyl acetate: acetic acid - 98:2. After separation, the plates were sprayed with 10% phosphomolybdic acid in etanol and heated at 120°C for 15 min (Gréen et al. 1978). This resulted in the appearance of bluish spots on a yellow-green background. The size and intensity of the spots depend on the concentration of prostaglandins in the sample.

RESULTS

In cells incubated with 100 μm of arachidonic acid, brown granules were present inside the cells at the sites of activity of prostaglandin synthetase. In the case of *T. rostrata*, the granules were more intensive than in *T. pyriformis* GL-C (Fig. 1). Omission of substrate (arachidonic acid) in control cells gave much less staining in all cells. A slight residual browning observed in controls may have arisen from endogenous fatty acids

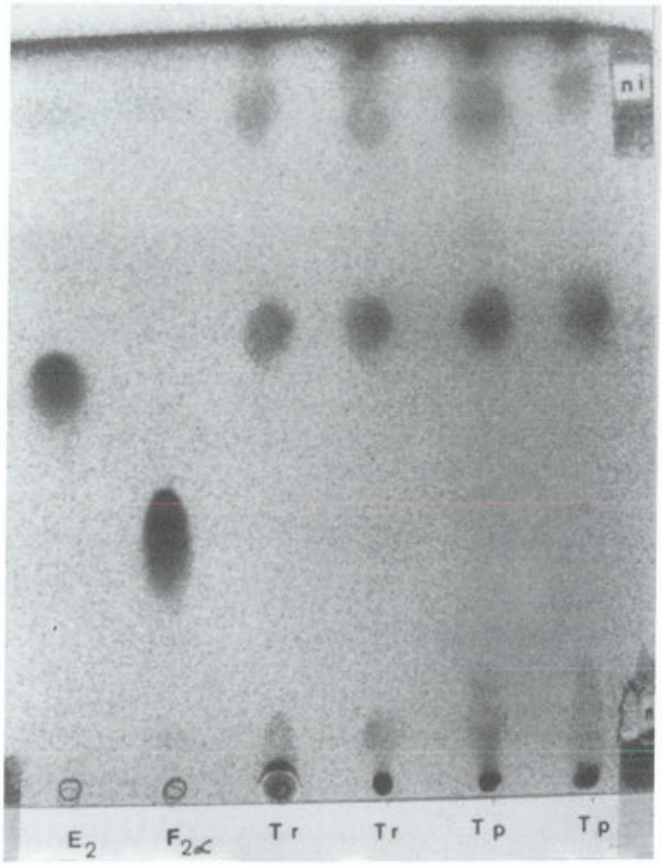

Fig. 2. The distribution of prostaglandins from *Tetrahymena* in solvent system ethyl acetate: acetic acid – 98:2; Tp – *Tetrahymena pyriformis* GL-C; Tr – *Tetrahymena rostrata; ni* – Fraction not identified

formed from phospholipids in the cell during the prolonged incubation (Janszen and Nugteren 1971). The results of separation of prostaglandins by thin-layer chromatography in ethyl acetate: acetic acid - 98:2 solvent system are presented in Fig. 2. In solvent system ethyl acetate: acetone: acetic acid - 80:10:10 there were differences only in the Rf value. The findings indicate that in both *Tetrahymena pyriformis* GL-C and *T. rostrata*, there are prostaglandins. The comparison of Rf values for standard and experimental prostaglandins suggests that PGE_2 is present. The other fractions of prostaglandins were not identified.

The concentration of PGE_2 in *T. pyriformis* GL-C is similar to that in *T. rostrata*.

DISCUSSION

The results suggest that we have identified in *Tetrahymena* the enzyme which is necessary for conversion of precursors into prostaglandins. The differences in intensity and colouring of granules between

T. rostrata and *T. pyriformis* GL-C in cytochemical investigations suggest that the activity of prostaglandin synthetase is lower in the free-living than in the parasitic form. The above mentioned findings suggest that prostaglandins may play an important role in *Tetrahymena*.

Metabolism of carbohydrates and lipids depends on the presence of prostaglandins (Hadaś 1987). Misra et al. (1983) suggested that pathogenicity of amoeba depends on presence of phospholipases functioning in the synthesis of prostaglandins.

Hadaś (1987) found differences in concentration of prostaglandins between pathogenic and non-pathogenic strains of *Acanthamoeba*. The pathogenic strains countaining more prostaglandin $F_{2\alpha}$ than non-pathogenic strains, but prostaglandin E_1 was present in higher concentration in a non-pathogenic strain. These findings were not observed in the case of *Tetrahymena*, although the experiments were carried out on free-living and parasitic species. This might be due to the fact that duration of growth phases differs in these species. While the cells were at a stationary phase of growth, they were probably at a different stage of this phase.

Although ciliates used in our experiments were isolated from renal organ of *Zonitoides nitidus*, they were maintained in long-term axenic culture. For this reason it is not an obligatory but a facultative parasite. Therefore there may be no differences (qualitative or quantitative) in prostaglandins between the investigated species. On the other hand, nobody has studied the effects of environmental changes on prostaglandins in protozoa. More precise and detailed experiments may give the answers to the present problems.

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ACTA PROTOZOOLOGICA

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Morphological Variation of the Ciliate *Trichodina pediculus* Ehrenberg, 1838. I. Parasitizing on Hydras

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Summary. Morphological variation between populations and between local groups of populations of a ciliate *Trichodina pediculus* Ehrenberg, 1838 from hydras, originating from Kortowo near Olsztyn and Szczęśliwickie Lake in Warszawa, was studied. There are no significant differences (with few exceptions) between groups of *T. pediculus* from different species of hydras and from Kortowo and Szczęśliwickie Lake. In the whole material, high variation between local groups of population, in almost all characters, was observed; while differences between populations were lacking or low. Variation of the ciliate *T. pediculus* from hydras differed from other species of trichodinas because of being higher between the local groups then between the populations.

Key words. Morphological variation, ciliate, *Trichodina pediculus*, parasite, hydras

INTRODUCTION

The ciliate *Trichodina pediculus* Ehrenberg, 1838 is a widely distributed species occurring mainly on hydras but also on tadpoles and various fish species (Kazubski 1965). Due to this diversity of hosts it is a good model for the study on variation. The present paper deals with morphological variation of the ciliates occurring on hydras. Variation of *T. pediculus* from other hosts will be presented in succeeding papers (Kazubski 1991a, b).

MATERIAL AND METHODS

The trichodinas used in the present investigation were collected in 1963-1964 from hydras, *Hydra vulgaris* and *Pelmatohydra oligactis*, from some water bodies in Kortowo near Olsztyn and in 1987 from *P. oligactis* from Szczęśliwickie Lake in Ochota district of Warsaw. The precise host species and dates of collection of each population are given in Table 1. The term "population" means here ciliates living on single host individual, according to Kazubski (1982).

Body and adhesive disc dimensions were measured on silver stained preparations after Klein. As an optimum sample size 30 ciliates from a population were measured, but due to low numerosity of trichodina populations occurring on single specimens of hydras, smaller samples were also used for counts. Measurements were taken according to previously adopted method (Kazubski 1979, 1982). The following characters were analyzed: (1) body diameter, (2) adhesive disc diameter with the border membrane, (3) adhesive disc diameter without the border membrane, (4) denticulate ring diameter, (5) number of denticles, and (6) denticle length. Additionally, in each population the mean length of the arch of the denticulate ring corresponding to a single denticle (width of denticle) was calculated according to the formula:

$$\frac{\text{denticulate ring diameter}}{\text{mean number of denticles}} \times \pi$$

The percentage difference between the smallest and the largest

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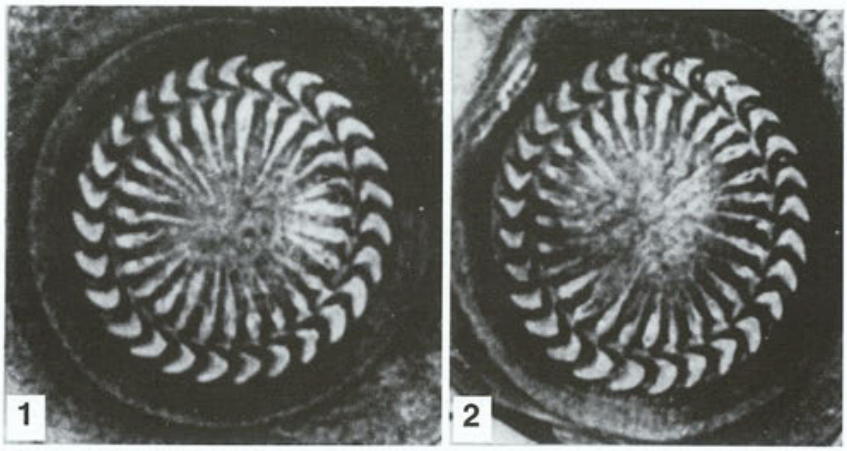
means within particular groups and between groups was counted in order to estimate primarily the results.

Variation was investigated using statistical methods, mainly the nested analysis of variance, with computation of expected mean squares and degrees of freedom, due to unequal sample size (according to Sokal and Rohlf 1981, Boxes 10.4 and .5). All characters were analyzed, but only three: (1) adhesive disc diameter without the border membrane, (2) denticulate ring diameter, and (3) number of denticles may be compared with previously published data. The choice of these characters as representative for trichodinas was proposed by Kazubski (1979).

RESULTS

Trichodina pediculus from hydras has a typical pattern of the adhesive disc and denticulate ring; its denticles have semilunar blades and long, rather wide, dagger-like rays (Figs. 1 and 2).



Figs. 1 and 2. *Trichodina pediculus* from hydras, 1000x

Values of the metric and the meristic characters of 24 populations of *T. pediculus* from hydras are given in Table 1. The table contains mean values and standard deviations of particular populations examined as well as groups of populations in different arrangement. The same concerning the whole material is also comprised. The mean values and standard deviation data for three characters: adhesive disc diameter without the border membrane, denticulate ring diameter, and number of denticles are presented in Fig. 3.

A rough analysis of the data, given in Table 1 and Fig. 3, has already shown great differences between mean values for particular characters of populations in the whole investigated material. These differences reach from 21.68 to 37.25 per cent of the minimum values of the characters. Also rather great differences, ranging from 13.8 to 22.93 per cent, exist between the means from the local groups of populations. Simultaneously, the differences within local groups of populations ranged from 0.06 to 19.3 per cent for particular characters, with an average from 5.2 per cent for the number of denticles, to 11.4 per cent for the diameter of the cell. Differences between means of particular characters of trichodinas from *Hydra* and *Pelmatohydra* collected in Kortowo reached 0.43-12.0 per cent while the differences between the material from Kortowo and Szczęśliwickie Lake 3.04-17.28 per cent.

The analysis of variance ascertained these observations. In the whole material embracing 24 populations (Table 2) the differences concerning five characters in ciliates from Kortowo and Szczęśliwickie Lake were insignificant, only the denticulate ring diameter differed at 3.7 per cent risk of error. Proportionally high variation was observed between local groups of populations; most characters showed significant differences with the risk of error lower than 1 per cent, denticulate ring diameter equal to 2.2 per cent and cell diameter - 2.5 per cent risk of error. Simultaneously, the variation between populations was not high; it was insignificant for the diameter of adhesive disc with border membrane, denticulate ring diameter and length of denticles, while significance at about 2.5 per cent risk of error was counted for the adhesive disc diameter, and 1.5 per cent for the number of denticles. The greatest differences concerned the cell diameter - the variance ratio 1.6 times overlapped the critical value at 1 per cent risk of error.

Similar results were obtained also in other configuration of the material, e.g. in the analysis of 15 populations from Kortowo (Table 3). Differences be-

tween groups of ciliates from various host species, *Hydra vulgaris* and *Pelmatohydra oligactis*, were insignificant for all characters except the number of denticles being different at 3.1 per cent risk of error. Differences between local groups of populations were rather high, attaining about 1 per cent risk of error or less than 1 per cent for five characters. Only the cell diameter showed no significant difference. The differences between populations were small, insignificant for most characters, except for the adhesive disc

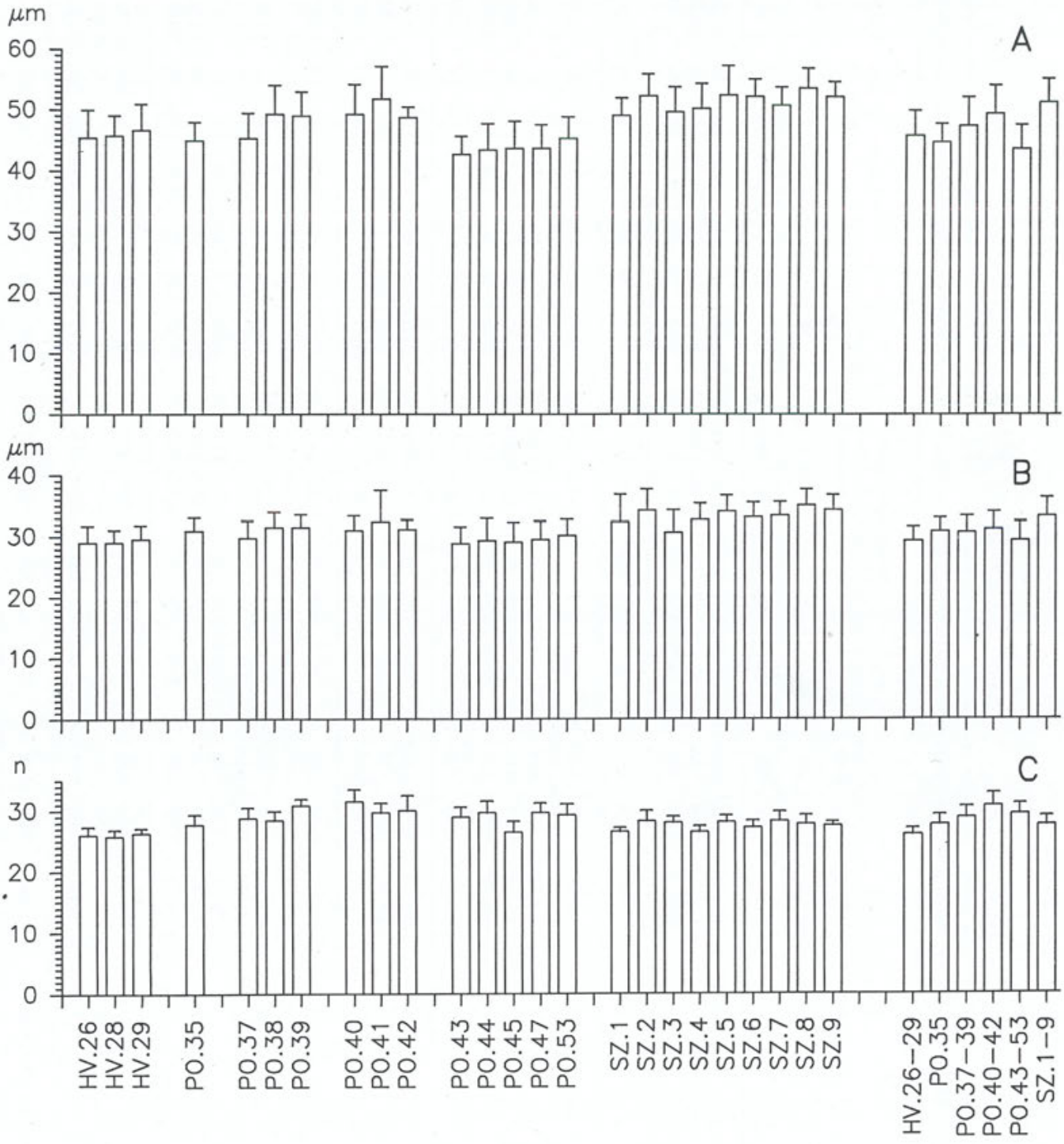


Fig. 3. Diagram of mean values and standard deviations of three characters: (A) diameter of adhesive disc, (B) diameter of denticulate ring, and (C) number of denticles in particular populations (on left) and local groups of populations (on right) of *Trichodina pediculus* from hydras

Table 1

Mean values (M) and standard deviations (SD) of main characters of examined populations of *Trichodina pediculus* from hydras (n = sample numerosity)

Host	Date	No. of popul.	Diameter (μm)												Number of denticles			Length of denticles (μm)			Width of denticles (μm)
			body			adhesive disc with border membrane			adhesive disc			denticulate ring									
			M	SD	n	M	SD	n	M	SD	n	M	SD	n	M	SD	n	M	SD	n	
Hydra vulgaris small water body in Kortowo	23.5.63	Hv. 26	81.60	10.60	25	51.59	3.97	23	45.41	4.49	29	29.07	2.71	30	26.00	1.32	24	15.75	1.42	10	3.51
	„	Hv. 28	81.88	7.95	17	51.56	3.79	16	45.71	3.35	21	29.25	2.03	22	25.75	1.07	20	16.40	2.07	5	3.57
	„	Hv. 29	90.52	8.05	21	51.26	4.01	27	46.61	4.35	31	29.65	2.18	31	26.30	0.84	30	15.94	1.90	23	3.54
	H.v.,	total	84.65	9.91	63	51.45	3.89	66	45.95	4.15	81	29.33	2.34	83	26.05	1.08	74	15.95	1.77	38	3.54
Pelmatohydra oligactis Lake Kortowo	20.7.64	Po. 35	72.67	5.65	30	52.07	3.55	28	44.90	3.03	30	30.90	2.23	30	27.70	1.60	30	15.59	1.33	22	3.50
	10.7.64	Po. 37	75.13	8.64	23	51.38	4.92	21	45.30	4.14	23	29.78	2.78	25	28.73	1.78	22	14.00	1.92	13	3.26
	„	Po. 38	82.18	9.15	22	54.29	3.93	21	49.27	4.84	22	31.48	2.51	22	28.43	1.51	14	15.08	1.38	12	3.48
	„	Po. 39	83.80	5.76	15	54.36	4.14	14	49.00	4.02	13	31.54	2.08	14	30.80	1.09	5	15.80	0.84	5	3.22
		total	79.83	8.96	60	53.21	4.53	56	47.64	4.72	58	30.80	2.64	61	28.88	1.75	41	14.73	1.68	30	3.35
	10.7.64	Po. 40	80.74	6.15	23	54.86	5.34	21	49.25	4.97	24	31.04	2.36	25	31.44	2.07	9	15.00	1.56	7	3.10
	„	Po. 41	88.17	9.24	6	57.67	5.72	6	51.83	5.42	6	32.42	5.10	6	29.67	1.53	3	17.00	–	1	3.43
	„	Po. 42	79.83	11.02	6	55.00	1.79	6	48.83	1.72	6	31.17	1.60	6	30.00	2.45	5	14.25	1.50	4	3.26
		total	81.86	7.97	35	55.39	4.96	33	49.61	4.68	36	31.28	2.82	37	30.71	2.14	17	14.92	1.38	12	3.20
	10.7.64	Po. 43	75.27	7.63	30	49.03	3.22	30	42.73	3.02	30	28.87	2.69	30	28.94	1.61	31	14.20	1.24	30	3.13
	„	Po. 44	73.28	9.66	29	49.67	4.48	30	43.47	4.33	32	29.34	3.69	32	29.67	1.92	30	14.13	1.73	24	3.11
	„	Po. 45	73.74	9.56	31	49.94	3.89	31	43.77	4.43	31	29.14	3.21	31	29.45	1.77	31	13.68	1.42	31	3.11
	„	Po. 47	77.13	9.90	30	48.35	4.09	31	43.71	3.93	31	29.53	3.01	31	29.63	1.63	30	13.26	1.64	25	3.13
	„	Po. 53	77.76	9.69	29	50.20	3.58	30	45.41	3.53	32	30.20	2.62	32	29.25	1.80	28	14.00	1.65	23	3.24
		total	75.42	9.36	149	49.43	3.89	152	43.83	3.94	156	29.43	3.07	156	29.39	1.74	150	13.85	1.54	133	3.15
	P.o.,	total	76.92	9.19	274	51.23	4.67	269	45.48	4.11	280	30.12	2.85	284	29.18	1.88	238	14.25	1.64	197	3.24
Mazurian Lake Region,		total	78.36	9.79	337	51.27	4.52	335	45.58	4.54	361	29.94	2.85	367	28.44	2.18	312	14.52	1.77	235	3.31

Table 1, cont.

Pelmatohydra oligactis Szczęśliwice	SZ. 1	81.86	8.76	7	58.50	7.07	2	49.22	2.89	3	32.42	4.50	6	26.50	0.71	2	–	–	–	3.84
	SZ. 2	88.00	10.59	18	59.00	3.76	15	52.39	3.65	18	34.25	3.48	18	28.25	1.77	16	18.20	0.45	5	3.81
	SZ. 3	81.09	9.44	11	57.00	3.79	6	49.75	4.20	8	30.71	3.64	7	28.00	1.00	3	–	–	–	3.44
	SZ. 4	83.09	11.38	22	56.17	3.76	18	50.35	4.13	20	32.75	2.64	20	26.47	0.94	17	16.62	1.56	13	3.89
	SZ. 5	87.18	10.26	22	56.67	5.50	12	52.62	4.87	16	34.09	2.61	17	28.10	1.10	10	17.00	0.89	6	3.81
	SZ. 6	92.00	5.69	6	57.00	3.74	4	52.33	2.94	6	33.14	2.41	7	27.17	1.17	6	18.00	–	1	3.83
	SZ. 7	91.08	8.60	12	55.92	3.65	12	50.92	2.96	13	33.46	2.19	13	28.30	1.57	10	16.67	1.36	6	3.71
	SZ. 8	95.67	11.20	6	59.33	3.06	3	53.75	3.30	4	35.12	2.59	4	27.75	1.50	4	17.00	–	1	3.98
	SZ. 9	93.00	9.16	3	59.00	3.46	3	52.33	2.52	3	34.33	2.52	3	27.50	0.71	2	18.00	–	1	3.92
	total	86.86	10.47	107	57.19	4.02	75	51.49	3.90	91	33.38	3.01	95	27.60	1.48	70	17.03	1.31	33	3.80
Total, total		80.41	10.59	444	52.35	4.98	410	46.77	5.01	452	30.65	3.20	462	28.28	2.09	382	14.83	1.91	268	3.40

Table 2

Three-level nested ANOVA table for six examined characters of 24 populations of *Trichodina pediculus* from hydras (whole material)

Source of variation	Degree of freedom	Diameter of body	Diameter of adhesive disc with border membrane	Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles	Length of denticles	Critical value df	Critical value F.01	Critical value F.05
Among groups of populations from Kortowo and Szczęśliwice	2–1=1	F_o 4.408 $F_{s'}$ 2.926 ns df' 3.07 ≈3	6.574 5.042 ns 3.86 ≈4	7.606 5.574 ns 3.65 ≈4	16.920 12.785 ≈3.7% 3.31 ≈3	0.241 0.173 ns 3.88 ≈4	4.416 4.039 ns 3.88 ≈4	(1,3) (1,4)	34.12 20.20	10.13 7.71
Among local groups of populations	6–2=4	F_o 5.266 $F_{s'}$ 4.218 ≈2.5% df' 15.04 ≈15	17.344 16.440 2.7x 9.504 ≈10	11.560 9.664 1.8x 12.25 ≈12	4.977 4.454 2.2% 10.75 ≈11	34.397 28.252 5.4x 12.90 ≈13	13.435 12.327 2.1x 9.96 ≈10	(4,10) (4,11) (4,12) (4.13) (4.15)	5.994 5.668 5.412 5.205 4.893	3.478 3.357 3.259 3.179 3.056
Among populations	24–6=18	F_o 3.122 1.6x	1.139 ns	1.776 ≈2.5%	1.359 ns	1.989 ≈1.5%	1.311 ns	(18.400)	1.98	1.63
Within populations	n–24	n=444	n=410	n=425	n=462	n=382	n=269			

Table 3

Three-level nested ANOVA table for six examined characters of 15 popualtions of *Trichodina pediculus* from hydras (from Kortowo)

Source of variation	Degree of freedom	Diameter of body	Diameter of adhesive disc with border membrane	Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles	Length of denticles	Critical value df	Critical value F.01	Critical value F.05
Among groups of populations from *Hydra* and *Pelmatohydra*	2–1=1	F_o 4.070 F_s' 3.629 ns df' 2.51 ≈3	0.006 0.004 ns 2.92 ≈3	0.032 0.022 ns 2.85 ≈3	0.696 0.490 ns 2.71 ≈3	14.341 ≈3.1% – –	3.798 ns – –	(1,3)	34.12	10.13
Among local groups of populations	5–2=3	F_o 2.772 F_s' 2.598 ns df' 9.54 ≈10	19.691 19.578 3.0x 9.66 ≈10	11.320 11.106 1.7x 9.73 ≈10	6.344 6.304 ≈1,5% 9.35 ≈9	8.13 1.2x – –	6.496 ≈1.1% – –	(3,9) (3,10) (3,11)	6.992 6.552 6.217	3.863 3.708 3.587
Among populations	15–5=10	F_o 3.614 1.5x	1.332 ns	2.356 ≈1.1%	1.189 ns	1.843 ns	1.542 ns	(10,200) (10,300)	2.41 2.38	1.88 1.86
Within populations	n–15	n=337	n=335	n=361	n=367	n=312	n=235			

Table 4

One-level nested ANOVA table for six examined characters of 9 populations of *Trichodina pediculus* from hydras (from Szczę=liwice)

Source of variation	Degree of freedom	Diameter of body	Diameter of adhesive disc with border membrane	Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles	Length of denticles	Critical value df	Critical value F.01	Critical value F.05
Among population	9–1=8	F_o 2.292 ≈3%	0.906 ns	1.077 ns	1.438 ns	2.271 ≈3.5%	1.179 ns df=6	(8,60) (8,80) (6,30)	2.823 2.743 3.473	2.097 2.057 2.421
Within populations	n–8	n=107	n=75	n=91	n=95	n=70	n=33			

diameter differing at about 1 per cent risk of error, and the cell diameter showing variance ratio 1.5 times overlapping 1 per cent risk of error.

The material from Szczęśliwickie Lake (Table 4) tested with one level analysis of variance showed also lack of significant differences for four characters examined while the cell diameter and the number of denticles significantly differed at about 3.0 per cent risk of error.

DISCUSSION

Trichodina pediculus is a peculiar ciliate which may live in different conditions: on the body surface of fresh-water hydras, on the body surface and gills of fishes as well as on the body surface of amphibian tadpoles. Populations living on hydras are usually small, counting from several to few dozens of very active specimens, quickly moving over the host body.

The present research has shown that there are no significant differences between groups of *T. pediculus* occurring on various species of hydras (*Hydra vulgaris*, *Pelmatohydra oligactis*) and in two fairly distant localities (Kortowo near Olsztyn and Szczęśliwickie Lake in Warsaw). Although in both cases mean values of one character examined showed significant difference (at not so high level), it should be regarded rather as casual. The highest variation was observed between local groups of populations - differences between means were usually significant, sometimes highly significant. The only exception was the cell diameter in the material from Kortowo. In this case, however, relatively high interpopulational variation was noted, being probably the result of deformation of the cell bodies during preparation. So, this character seems to be of little value. The variation of other characters was rather low, while the adhesive disc diameter with border membrane, denticulate ring diameter and length of denticles showed only insignificant differences.

Thus, the variation of *T. pediculus* from hydras represents the pattern different from that observed in other species of trichodinas (Kazubski 1982, 1988) because for the first time the variation between local groups was higher than the interpopulational variation. This is probably due to the fact that *T. pediculus* forms "open" populations on hydras, characterized by great exchange of specimens. Intensity of this exchange is probably so high that differences between particular populations occurring on single host specimens are obliterated. So, in this case we have probably to do with formation of larger populations, embracing all specimens of *T. pediculus* living in a local environment.

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Morphological Variation of the Ciliate *Trichodina pediculus* Ehrenberg, 1838. II. Parasitizing on Tadpoles

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Summary. Morphological variation of the ciliate *Trichodina pediculus* Ehrenberg, 1838 between populations from tadpoles of *Rana esculenta* and *Bufo bufo*, from different localities in Kortowo near Olsztyn, was investigated using statistical methods. No differences (with the exception of denticulate ring diameter) between mean values of examined characters of *T. pediculus* from two species of tadpoles, originating from the same pond and collected at the same time, were noted. The analysis of variance has shown fairly small differences between means of particular local groups of populations and much greater differences between populations.

In some populations of *T. pediculus* from tadpoles the teratological changes, concerning the shape of denticles and the structure of denticulate ring, were observed.

In comparison with *T. reticulata*, parasitizing on tadpoles (Kazubski 1988) in the same conditions, *T. pediculus* shows many differences.

Key words. Morphological variation, ciliate, *Trichodina pediculus*, parasite, teratology, tadpoles

INTRODUCTION

The ciliates *Trichodina pediculus* Ehrenberg, 1838, typical parasites of hydras and fishes, may occur also on amphibian tadpoles. A study of their variation is especially interesting due to the fact that these ciliates form temporary populations on tadpoles, being renewed each year by protozoans, occurring in the same pond on hydras and fishes.

MATERIAL AND METHODS

The trichodinas used in the present investigation were collected in 1963-1964 from tadpoles of *Rana temporaria* and *Bufo bufo* from some water bodies in Kortowo near Olsztyn. The precise host species, date and place of collection of each population are given in Table 1.

The concept of the population, methods of preparation of the material, measuring of ciliates and statistical elaboration of the data have been presented in the Ist part of this paper (Kazubski 1991a).

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RESULTS

Trichodina pediculus from tadpoles has a typical pattern of the adhesive disc and denticulate ring; its denticles have semilunar blades and long, rather wide, dagger-like rays (Figs. 1 and 2).

The values of the metric and the meristic characters of 33 populations and seven local groups of populations of *T. pediculus* from tadpoles are given in Table 1. The mean values and standard deviations for three characters are presented in Fig. 3.

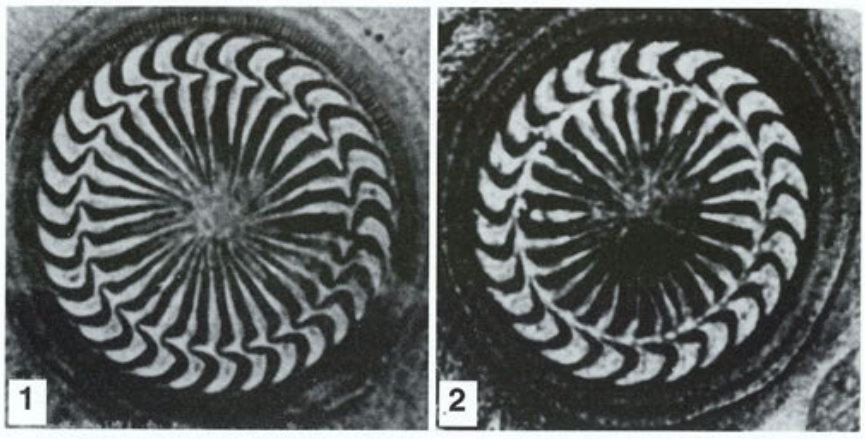
Analysis of the material comprised in Table 1 and Fig. 3, has shown very high differentiation between mean values obtained for particular populations in the

whole material examined. These differences reach from 30.1 to 66.2 per cent of the minimum values for the metric data and 8.25 per cent for the number of denticles. Differences between means from local groups of populations are smaller and reach from 7.6 to 17.2 per cent for the metric character and 4 per cent for the number of denticles. In particular local groups of populations these differences ranged from 2 to 52.8 per cent for particular characters, with the average 4 per cent for the number of denticles and from 9.6 to 24.1 per cent for remaining metric characters.

Only small differences, ranging from 0.9 to 3.4 per cent, were observed between means of particular characters in trichodinas from two species of tadpoles, *Rana temporaria* and *Bufo bufo*, originating from the same pond and collected at the same time. The analysis of variance (Table 2) ascertained the above observations showing lack of significant differences in almost all the characters in the compared groups of populations. Only the denticulate ring diameter differed but not so greatly high level. This result allows to treat the group of populations from tadpoles of *B. bufo* in the same way as those from *R. temporaria*.

The analysis of variance performed on this material (Table 3) has shown fairly small differences between means of local groups of populations and much greater differences between particular populations. Differences between means of groups were statistically insignificant for the cell and the adhesive disc diameters, while the diameter of the adhesive disc with border membrane, denticulate ring diameter and the number of denticles may be estimated with the risk of error equal to 0.9-2.5 per cent. The greatest differences concerned the length of denticles - the variance ratio was twice as large as the critical value at 1 per cent risk of error. Interpopulational variation was maintained at a fairly high level. Significance of differences between means of the cell diameter, diameter of the adhesive disc with border membrane and without it, diameter of denticulate ring and length of the denticles may be estimated with the risk of error not overlapping 1 per cent. Intrapopulational variation was statistically insignificant only in the case of the number of denticles.

The significance of differences between means for populations of *T. pediculus* from *R. temporaria* and *B. bufo* originating from two ponds (Kaczy Dołek and Stary Dwór) in Kortowo near Olsztyn was also tested. The differences between means of these groups amounted from 1.0 to 5.0 per cent for the cell, adhesive disc and denticulate ring diameters, 1.2 per cent for the number of denticles and 13.1 per cent for the length of denticles. The analysis of variance (Table 4) has shown complete lack of significance between groups of trichodinas from both ponds and between groups of local populations with the exception of denticulate ring diameter. In this material differences between populations appear to be the main source of variation. For most characters these differences were moderately high and the variance ratio 1-2 times overlapped the critical values at 1 per cent risk of error. As the number of denticles is concerned the significance of differences may be estimated at about 5 per cent risk of error. The analysis of the material only from *R. temporaria* tadpoles gave the same result.



Figs. 1 and 2. *Trichodina pediculus* from tadpoles, 1000x

Among *T. pediculus* occurring on tadpoles numerous specimens showing teratological changes were

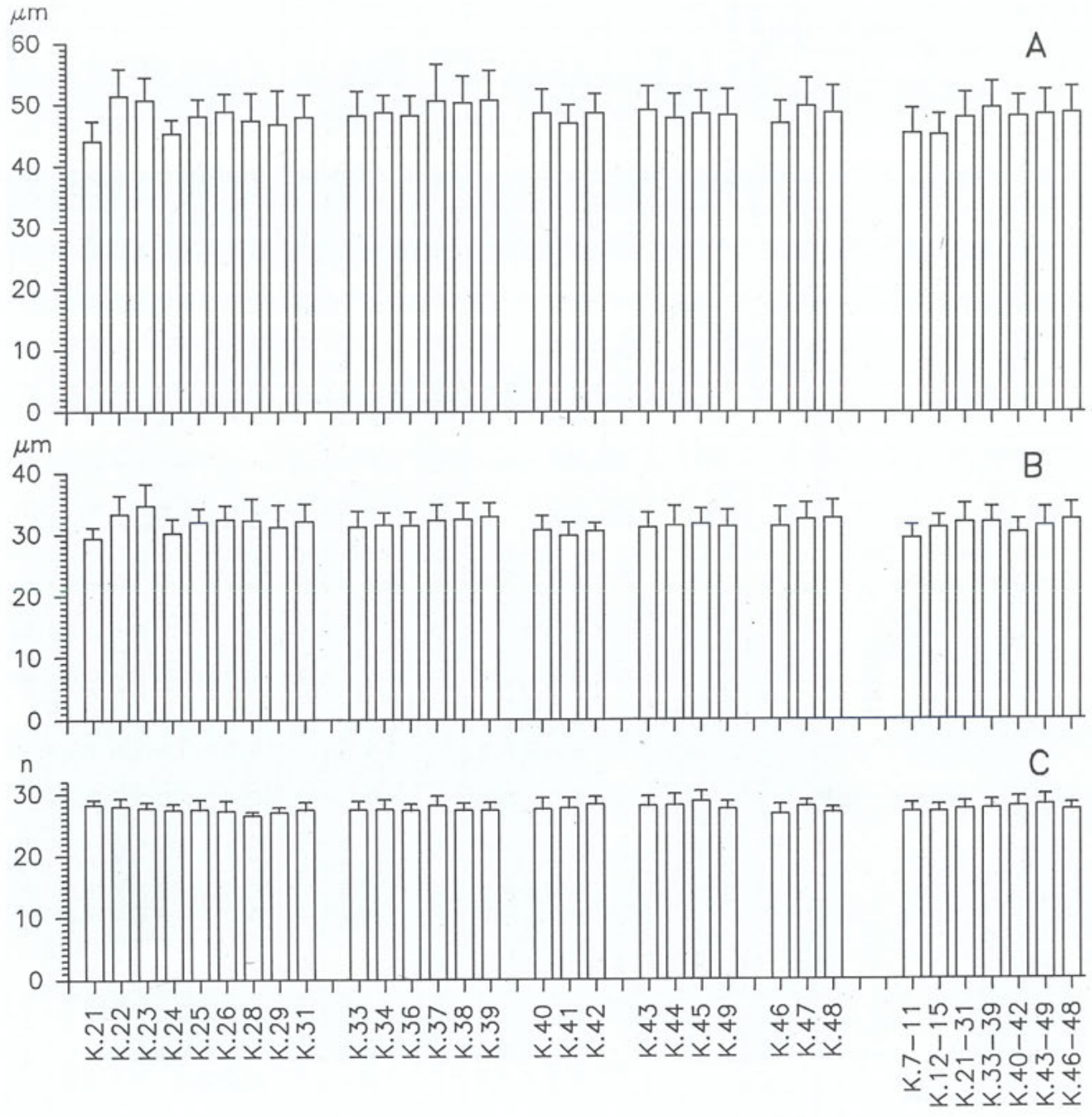


Fig. 3. Diagram of mean values and standard deviations of three characters: (A) diameter of adhesive disc, (B) diameter of denticulate ring, and (C) number of denticles in particular populations (on left) and local groups of populations (on right) of *Trichodina pediculus* from hydras

observed (see Table 1). The changes concerned the shape of the denticles and the structure of the denticulate ring. In populations No. 33, 34 and 37-39, collected on 21st and 22nd May 1964, these changes were observed in 6-10 per cent of specimens. More frequently, in 16.7 per cent of specimens, these changes were observed in population No. 40, in 36.7 per cent of ciliates from the population No. 41, and 34.2 per cent in the population No. 42, collected on 30th May 1964. The last group of populations originated from tadpoles

Table 1

Mean values (M) and standard deviations (SD) of main characters of examined populations of *Trichodina pediculus* from tadpoles (n = sample numerosity)

Host	Date	No. of popul.	Diameter (μm)												Number of denticles			Length of denticles (μm)			Width of denticles (μm)	Teratology %
			body			adhesive disc with border membrane			adhesive disc			denticulate ring										
			M	SD	n	M	SD	n	M	SD	n	M	SD	n	M	SD	n	M	SD	n		
Rana temporaria	23.5.63	k. 7	71.60	11.73	5	50.20	3.11	5	44.83	1.94	6	29.50	1.48	6	27.00	1.90	6	15.25	0.96	4	3.43	
	„	k. 8	66.67	7.23	3	54.50	0.71	2	50.67	0.58	3	31.67	1.53	3	26.67	0.58	3	16.50	2.12	2	3.73	
	10.6.63	k. 9	78.00	—	1	47.00	—	1	41.00	—	1	28.50	—	1	27.00	—	1	15.00	—	1	3.32	
	„	k. 10	56.50	—	2	45.00	—	2	39.50	—	2	26.00	—	2	28.00	—	2	14.00	—	1	2.92	
	„	k. 11	86.33	10.41	3	51.67	7.02	3	46.33	4.51	3	29.50	2.29	3	26.67	1.53	3	18.50	—	2	3.47	
		total	72.00	12.76	14	50.15	4.69	13	45.33	4.15	15	29.40	2.20	15	27.00	1.36	15	16.00	2.40	10	3.42	
Rana temporaria	26.6.63	k. 12	—	—	—	—	—	—	43.00	—	1	29.00	—	1	27.00	—	1	16.00	—	1	3.37	
	„	k. 14	81.00	—	2	56.00	—	2	48.50	—	2	32.00	—	2	26.50	—	2	20.50	—	2	3.79	
	„	k. 15	71.43	9.62	7	53.86	3.24	7	44.43	3.51	7	31.21	2.23	7	27.14	1.35	7	15.86	2.97	7	3.61	
		total	73.56	9.36	9	54.33	3.00	9	45.10	3.41	10	31.15	2.00	10	27.00	1.15	10	16.80	3.19	10	3.62	
Rana temporaria Kaczy Dołek (K.D.)	14.5.64	k. 21	58.63	5.64	5	48.80	4.09	5	44.00	3.32	5	29.40	1.82	5	28.20	0.84	5	12.33	2.08	3	3.53	
	„	k. 22	75.00	13.34	12	56.85	5.13	13	51.38	4.46	13	33.35	2.96	13	28.00	1.35	13	17.44	2.24	9	3.74	
	„	k. 23	83.11	12.86	9	55.33	3.61	9	50.67	3.77	9	34.78	3.48	9	27.75	0.89	8	17.29	1.60	7	3.94	
	„	k. 24	72.65	9.87	17	50.07	2.34	15	45.28	2.24	16	30.28	2.31	18	27.36	1.01	14	14.85	1.77	13	3.48	
	„	k. 25	72.79	8.99	19	52.35	2.71	17	48.15	2.70	20	32.12	2.11	20	27.50	1.56	14	15.33	1.11	15	3.67	
	„	k. 26	74.06	7.57	18	53.95	3.46	21	48.88	2.91	25	32.50	2.22	27	27.28	1.71	29	16.61	2.21	23	3.75	
	„	k. 28	71.50	10.20	8	52.25	5.36	8	47.37	4.50	8	32.37	3.45	8	26.50	0.55	6	15.40	1.95	5	3.84	
	„	k. 29	67.12	11.23	17	51.00	6.13	19	46.83	5.43	23	31.30	3.56	23	26.94	0.85	18	15.67	1.68	15	3.65	
	„	k. 31	67.67	10.17	27	52.14	4.20	29	47.93	3.73	30	32.20	2.80	30	27.40	1.25	30	15.93	1.94	27	3.69	
		total	71.45	11.02	132	52.60	4.60	136	47.97	4.09	149	32.05	2.94	153	27.39	1.31	137	15.91	2.05	117	3.68	

Table 1 (cont.)

Rana	21.5.64	k. 33	75.63	9.05	32	54.97	3.91	30	48.22	3.98	32	31.27	2.59	32	27.37	1.45	32	18.34	2.06	32	3.59	6.25
temporaria	„	k. 34	74.36	10.41	28	54.21	4.00	29	48.75	2.79	32	31.63	1.96	31	27.52	1.57	31	18.03	1.84	29	3.61	6.5
Stary Dwór	22.5.64	k. 36	72.06	7.49	18	53.45	3.22	20	48.25	3.18	20	31.53	2.18	20	27.32	0.95	19	17.73	1.42	11	3.63	–
(S.D.)	„	k. 37	72.96	8.51	28	55.90	4.43	29	50.65	5.98	30	32.33	2.50	30	28.07	1.53	29	17.41	2.10	29	3.62	10.0
	„	k. 38	71.10	9.54	31	57.61	4.94	26	50.32	4.40	31	32.48	2.72	31	27.29	1.22	31	19.00	2.10	28	3.74	6.5
	„	k. 39	74.21	10.10	29	58.83	4.41	30	50.73	4.92	30	32.95	2.24	30	27.33	1.21	30	19.67	1.92	30	3.79	6.7
		total	73.48	9.33	166	55.94	4.55	164	49.54	4.29	175	32.05	2.43	174	27.49	1.37	172	18.44	2.09	159	3.64	
	30.5.64	k. 40	72.97	11.16	30	54.80	4.68	30	48.65	3.86	30	30.77	2.35	30	27.47	1.79	30	19.00	1.84	30	3.52	16.7
	„	k. 41	71.83	7.87	30	54.23	3.85	30	47.00	2.97	30	29.82	2.16	30	27.63	1.67	30	18.27	1.55	30	3.39	36.7
	„	k. 42	78.50	9.96	30	57.40	3.57	30	48.63	3.17	30	30.60	1.32	30	28.20	1.24	30	18.60	1.83	30	3.41	34.2
		total	74.43	10.08	90	55.48	4.25	90	48.09	3.41	90	30.39	2.17	90	27.77	1.60	90	18.66	1.76	90	3.44	
	8.6.64	k. 43	73.79	11.39	24	53.73	3.99	26	49.10	3.96	29	31.14	2.48	29	28.00	1.54	29	16.13	1.85	15	3.49	
	„	k. 44	80.86	10.90	28	54.39	4.82	28	47.76	4.02	29	31.53	3.11	29	28.07	1.80	27	17.90	2.49	20	3.53	
	„	k. 45	74.52	10.14	29	53.73	3.87	30	48.51	3.82	33	31.76	2.53	33	28.69	1.67	32	17.55	1.45	29	3.48	
	„	k. 49	78.10	12.12	30	54.81	4.48	27	48.29	4.22	31	31.32	2.72	31	27.50	1.25	30	16.54	1.68	22	3.58	1·
		total	76.93	11.36	111	54.16	4.26	111	48.42	3.99	122	31.45	3.01	122	28.08	1.61	118	17.13	1.95	86	3.52	
	R.t., S.D.	total	74.76	10.25	367	55.28	4.44	365	48.85	4.04	387	31.47	2.65	386	27.74	1.52	380	18.16	2.06	335	3.55	
Bufo bufo	8.6.64	k. 46	90.25	9.18	4	55.00	2.65	3	47.00	3.61	3	31.33	3.21	3	26.67	1.53	3	16.33	1.16	3	3.69	
Stary Dwór	„	k. 47	74.62	11.54	8	56.43	5.97	7	49.75	4.59	8	32.50	2.67	8	27.86	1.07	7	17.14	1.95	7	3.66	1·
	„	k. 48	66.71	6.78	7	53.00	3.89	8	48.67	4.44	9	32.67	2.95	9	26.87	0.84	8	16.00	1.67	6	3.82	
		total	75.00	12.63	19	54.67	4.72	18	48.85	4.28	20	32.40	2.76	20	27.22	1.11	18	16.56	1.71	16	3.74	
R.t. B.b., S.D.		total	74.77	10.36	386	55.26	4.45	383	48.85	4.05	407	31.52	2.66	406	27.63	1.44	535	18.09	2.07	351	3.57	
R.t., K.D. + S.D.		total	73.88	10.55	499	54.56	4.64	501	48.61	4.07	536	31.64	2.74	539	27.64	1.48	517	17.58	2.28	452	3.59	
R.t. + *B.b.*, K.D. + S.D.		total	73.92	10.62	518	54.56	4.64	519	48.61	4.07	556	31.66	2.68	559	27.63	1.47	535	17.54	2.27	468	3.60	
Total, total			73.87	10.64	541	54.45	4.66	541	48.47	4.12	581	31.60	2.74	584	27.60	1.46	560	17.50	2.30	488	3.59	

Table 2

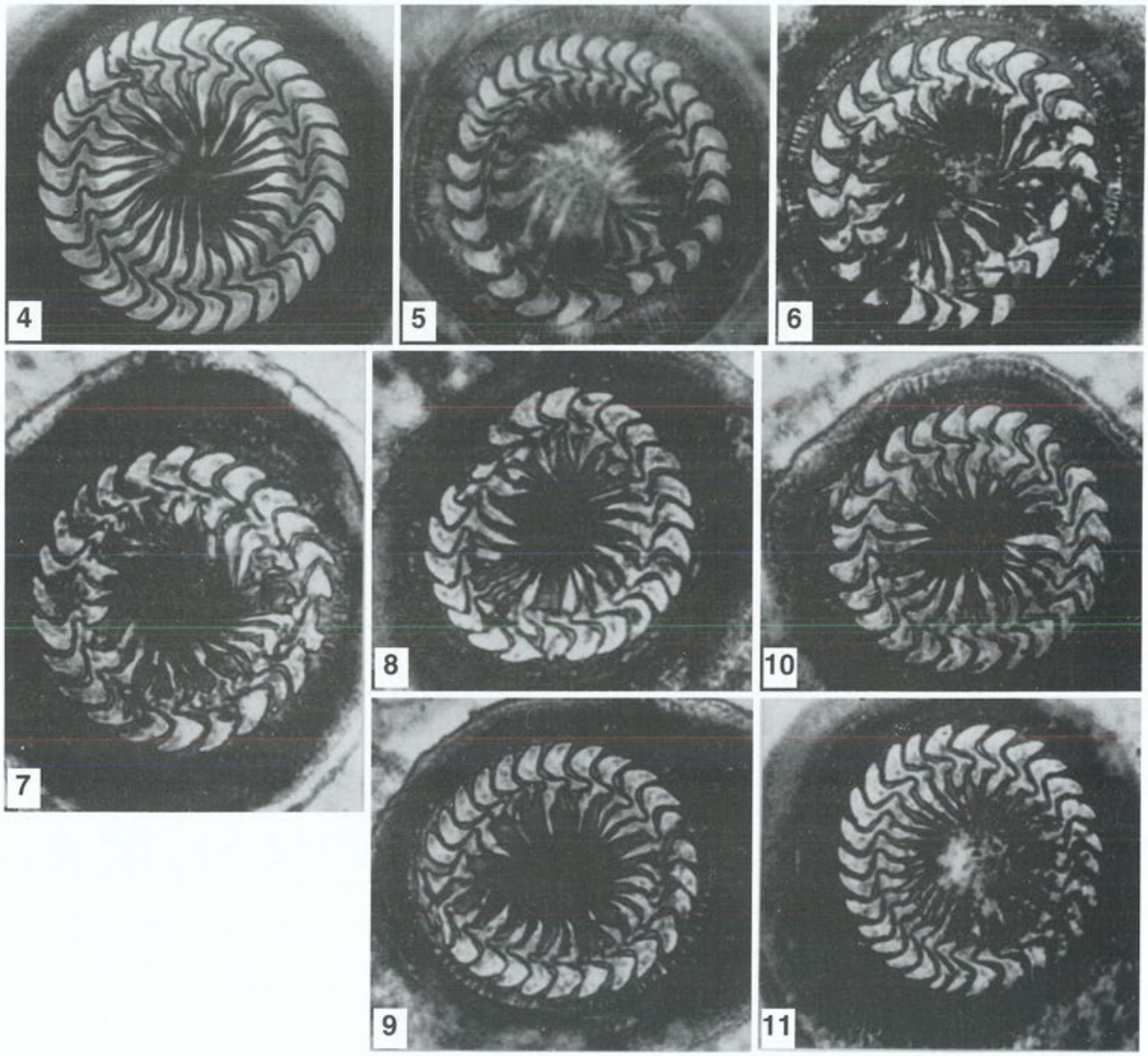
Two – level nested ANOVA table for six examined characters of 7 populations of *Trichodina pediculus* from tadpoles *R. temporaria* and *B. bufo*

Source of variation	Degree of freedom	Diameter of body	Diameter of adhesive disc with border membrane	Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles	Length of denticles	Critical value df	Critical value F. 01	Critical value F. 05
Among groups of populations from *R. temporaria* and *B. bufo*	2−1=1	F_0 0.132 ns	0.291 ns	0.364 ns	7.237 ≈3.6%	2.125 ns	0.492 ns	(1,5)	16.26	6.608
Among populations	7−2=5	F_0 3.809 1.6x	0.716 ns	0.535 ns	0.237 ns	2.315 ns	2.574 ≈4%	(5,80) (5,120)	3.564 3.480	2.486 2.447
Within populations	n−7	n=130	n=129	n=135	n=135	n=129	n=95			

Table 3

Two – level nested ANOVA table for six examined characters of 33 populations of *Trichodina pediculus* from tadpoles (whole material)

Source of variation	Degree of freedom	Diameter of body	Diameter of adhesive disc with border membrane	Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles	Length of denticles	Critical value df	Critical value F. 01	Critical value F. 05
Among local groups of populations	7−1=6	F_0 1.020 ns F_s' – df' –	3.396≈1.5% – –	2.278 ns – –	3.714 ≈0.9% – –	3.057 ≈2% – –	8.503 8.345 2.3x 25.58 ≈26	(6,25) (6,26)	3.62 3.59	2.49 2.47
Among populations	32 or 33 −7=	F_0 3.136 1.7x df=25	3.331 1.8x df=25	2.474 1.3x df=26	1.889 ≈1.04x df=26	1.312 ns df=26	3.284 1.8x df=26	(25,500)	1.81	1.53
Within population	n−32 or 33=	n=541	n=541	n=481	n=584	n=560	n=488			



Figs. 4-11. *Trichodina pediculus* from tadpoles. Teratology. Explanation see text

maintained several days in an aquarium placed in well insolated room. The ciliates from populations Nos. 40-42 had small denticulate rings attaining mean diameter of 30.77, 28.82 and 30.6 μm respectively. Single cases of teratological changes were observed also in other populations of *T. pediculus*, e.g. No. 49 from *R. temporaria* and No. 47 from *Bufo bufo*.

These specimens displayed teratological changes of various degrees. The smallest changes manifested by deformation and shortening of the radii of the denticles which had however normal blade (Fig. 4). In many specimens the changes involved both radius and blade, but deformation of the radius was more frequent. Sometimes the denticulate ring was broken and its free ends overlapped each other (Figs. 5 and 6). Usually the denticles were the most deformed in the place of fracture (Figs. 7-9). Other type of deformation of the denticles were manifested by their smaller width - single denticles or their groups were clearly narrower than the normal ones (Fig. 11). It is especially well visible in the central part of the denticles. Sometimes, elements being probably remnants of underdeveloped denticles were visible beside the denticulate ring (Fig. 10).

Table 4

Three – level nested ANOVA table for six examined characters of 25 populations of *Trichodina pediculus* from tadpoles *R. temporaria* and *B. bufo* from ponds Kaczy Dołek and Stary Dwór

Source of variation	Degree of freedom	Diameter of body	Diameter of adhesive disc with border membrane	Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles	Length of denticles	Critical value df	F. 01	F. 05
Among groups of populations from Kaczy Dołek and Stary Dwór	2−1=1	F_0 4.036 ns F_S – df' –	9.632 ns –	1.610 ns –	0.521 ns –	1.131 ns –	7.529 5.477 ns 2.496 ≈2	(1,2) (1,3)	98.50 34.1	18.51 10.1
Among local groups of populations	5−2=3	F_0 0.819 F_S 0.779 ns df' 19.16 ≈19	1.072 1.025 ns 19.39 ≈19	1.360 1.319 ns 19.20 ≈19	4.126 4.032 ≈2.1% 19.19 ≈19	2.895 2.821 ns 18.45 ≈18	4.312 3.994 ns 18.92 ≈19	(3,18) (3,19) (3,20)	5.09 5.01 4.94	3.16 3.13 3.10
Among populations	25−5=20	F_0 3.235 1.7x	3.841 ≈2.01x	2.498 1.3x	2.089 1.06x	1.624 ≈5%	3.621 1.9x	(18,500)	1.97	1.62
Within populations	n−25	n=518	n=519	n=556	n=559	n=535	n=468			

Table 5

Mean values of six examined characters of *Trichodina pediculus* from hydras and tadpoles of *R. temporaria* and *B. bufo* from Kortowo

	Diameter of body		Diameter of adhesive disc with border membrane		Diameter of adhesive disc		Diameter of denticulate ring		Number of denticles		Length of denticles	
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
Trichodinas from hydras populations Hv 26–29, Po 35–53	78.36	9.79 n=37	51.27	6.52 335	45.58	4.54 361	29.94	2.85 367	28.44	2.18 312	14.52	1.77 235
Trichodinas from tadpoles populations k 21–45, 49 and k 46–48	73.92	10.62 n=18	54.56	4.64 519	48.61	4.07 556	31.66	2.74 559	27.63	1.47 535	17.54	2.27 468
Differences between means		6.01%		6.42%		6.65%		5.58%		2.93%		20.08%

DISCUSSION

The investigation carried out has shown that there are no important differences between *T. pediculus* from tadpoles of *R. temporaria* and *B. bufo* - most differences between them were statistically insignificant. Only the differences between diameters of the denticulate ring were significant but at a fairly low level.

The investigation carried out in the whole material has shown fairly small variation between local groups - the differences between means were statistically insignificant or significant at a fairly low level. The interpopulational variation was higher in relation to metric characters, being however not observed in the case of the meristic one - the number of dentic-les. Similar results were obtained when more uniform part of the material was elaborated, namely the populations originating from two ponds - Kaczy Dołek and Stary Dwór in Kortowo. The differences between means from both ponds, as well as differences between local groups of populations were statistically insignificant, while interpopulational differences were statistically significant at fairly high level, except for the number of denticles showing the risk of error at the margin of significance.

The material collected and the methods of its elaboration used in the present investigation allow to compare the trichodinas *T. pediculus* from tadpoles with those from hydras described in the Ist part of this paper (Kazubski 1991). For methodical purposes and in order to obtain more uniform material of *T. pediculus* from hydras, the material from Szczęśliwice (Warszawa) and some not numerous populations from tadpoles collected in 1963 were rejected.

Means, standard deviations and numerosity of samples for all characters examined of *T. pediculus* from hydras and tadpoles are presented in Table 5. The differences between means are not great, attaining 5.9-6.7 per cent for the cell dimensions, adhesive disc and denticulate ring diameters, 2.9 per cent for the number of denticles and 20.1 per cent for the length of denticle in relation to the smallest value. In trichodinas from tadpoles dimensions of the adhesive disc, denticulate ring and length of the denticle were larger and the number of denticles smaller than in the ciliates from hydras. The results of the analysis of variance are presented in Table 6 with three levels of groups discerned and local groups treated as being of equal rank. Such an assumption is justified by earlier investigation carried up on this material which has shown that any grouping at higher level has only little bearing on variation of the ciliates examined. The analysis of

Table 6

Three – level nested ANOVA table for six examined characters of 40 populations of *Trichodina pediculus* from hydras and tadpoles

Source of variation	Degree of freedom	Diameter of				Number of denticles	Length of denticles	Critical value		
			body	adhesive disc with border membrane	adhesive disc	denticulate ring			df	F. 01	F. 05
Among groups of populations from hydras and tadples	2 – 1 = 1	F_0	4.471	7.902	10.215	12.506	1.460	15.361	(1,6)	13.75	5.99
		F_s'	3.549 ns	7.362 ≈3.6%	7.858 ≈2.7%	9.797 ≈1.7%	1.023 ns	10.057 ≈1.3%	(1,7)	12.2	5.59
		df'	6.90 ≈7	5.95 ≈6	7.34 ≈7	7.15 ≈7	7.83 ≈8	7.59 ≈8	(1,8)	11.30	5.32
Among local groups of populations	10 – 2 = 8	F_0	2.917	5.269	5.089	4.162	23.310	9.539	(8,28)	3.36	2.36
		F_s'	2.769 ≈2.5%	5.079 1.5x	4.882 1.45x	4.030 1.2x	22.913 6.9x	9.362 2.8x	(8,29)	3.33	2.35
		df',	28.76 ≈29	28.97 ≈29	28.37 ≈28	27.65 ≈28	28.58 ≈29	29.47 ≈29	(3,30)	3.30	2.33
Among populations	40 – 10 = 30	F_0	3.397 2.0x	3.048 1.8x	2.439 1.4x	1.762 1%	1.695 ≈1.1%	3.088 1.8x	(30,800)	1.73	1.47
Within population	n – 40		n = 855	n = 854	n = 917	n = 926	n = 847	n = 703			

variance has shown that the differences between groups of trichodinas from hydras and tadpoles are statistically insignificant (cell diameter, number of denticles) or their significance is at rather low level with the risk of error equal to 3.6-1.3 per cent. So, almost the whole variation in the material examined has the source either in local groups or in interpopulational differences. In both cases the significance of differences may be estimated with the risk of error close to or smaller than 1 per cent. Out of the characters examined the number of denticles is the most outstanding due to the lack of statistically significant differences between groups from populations from various hosts and very high variation between local populations (coefficient of variation almost 7 times overlapped the value F_o at 1 per cent risk of error).

This investigation allows to estimate bearing of the host on the morphology of trichodinas. Populations of *T. pediculus* on tadpoles have temporary character, disappearing in the moment of leaving of the aquatic environment by freshly metamorphosed frogs and toads. Thus, each year they are renewed by ciliates occurring on hydras and fishes. In this situation the changes and their range, as observed in trichodinas occurring on tadpoles, are the result of the influence of the host. In the case of *T. pediculus* occurring on tadpoles, the changes are small or insignificant, so, it may be stated that bearing of the host on the morphology of these trichodinas is rather small.

Similar problem has been analyzed in the case of *T. reticulata* from crucian carp and tadpoles (Kazubski 1982, 1988) but quite opposite results were obtained. In this case great influence of the tadpole host on the morphology of ciliates has been observed, manifested in the decrease of the cell, adhesive disc and denticulate ring diameters as well as in diminishing of the denticle length, especially in the material collected at the beginning of summer. These changes were accompanied, however, by rather stable number of denticles. Quick increase of the numerosity of *T. reticulata* populations on tadpoles was supposed as the cause of these changes (Kazubski 1988).

Teratological changes are rather rarely observed in trichodinas. Most frequently they are observed in single specimens being usually manifested by deformation of inner radii of the denticles. This deformation appear during formation of the denticles after cell division (Kazubski 1967) when growing radii meet with remnants of not yet resorbed, disintegrating denticles of the old ring. Changes observed in *T. pediculus* and described in the present paper, seem to have another character. In general they concern the whole ring, frequently manifesting in its breaking with accompanying deformation of the denticles, especially at free ends of a broken ring. These changes were very frequent, embracing about 10 per cent of ciliates in a group of population collected on 21st-22nd May and up to 36.7 per cent in populations collected on 30th May. Moreover, single teratological specimens were noted in other populations examined (see Table 1). These changes were observed at the end of May 1964 characterized by relatively high temperature of the water in which the tadpoles infected with *T. pediculus* lived.

The analysis of data from Table 1 has shown that these populations were characterized by the smallest diameters of the adhesive disc and of the denticulate ring but the average number of the denticles. This caused either strong deformation of the denticles in any place and subsequent breaking of the ring or more dense packing of the denticles in the ring, leading to their narrowing at least in some parts of the ring (Fig. 9). The described phenomenon shows that the seasonal changes in trichodinas concern mainly the cell dimensions while the number of denticles rests under the control of genetic factors being thus less variable and changing more slowely and to smaller extent, giving as a result defined disturbances in their morphogenesis.

It ought to be noted that in *T. reticulata* found on tadpoles in the same place and time as *T. pediculus*, no teratological changes have been observed (Kazubski 1988). This observation, as well as observation on different morphological reaction of *T. reticulata* and *T. pediculus* in the same situation - passage to tadpoles, proves that various species of trichodinas may display different types of reaction to changes in their environment.

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Morphological Variation of the Ciliate *Trichodina pediculus* Ehrenberg, 1838. III. Parasitizing on Crucian Carp *(Carassius carassius* (L.)) from Small Ponds in Kortowo (Olsztyn)

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Summary. Morphological variation between populations of the ciliate *Trichodina pediculus* Ehrenberg, 1838 from crucian carp (*Carassius carassius* (L.)) originating from small ponds in Kortowo (Olsztyn) is maintained at a rather low level. Simultaneously, great seasonal differences in dimensions of the cell and other morphotic elements as well as in the number of denticles were observed in *T. pediculus* from crucian carps collected in spring and summer.

Summing up of the variation in *T. pediculus* from hydras, tadpoles and crucian carps (Kazubski 1991a, b and present paper) has shown that the differences between mean values of metric characters are placed at a fairly low level, while the number of denticles does not show statistically significant differences. Proper analysis of the data has shown, that these differences display seasonal character being independent of the host species.

Key words. Morphological variation, ciliate, *Trichodina pediculus*, parasite, fish, *Carassius carassius*.

INTRODUCTION

The ciliates *Trichodina pediculus* Ehrenberg, 1838, typical parasites of hydras, occur often on fish. They were found also in small ponds in Kortowo (Olsztyn), in the same place where this species was noted on hydras and tadpoles (Kazubski 1991a, b).

MATERIAL AND METHODS

The trichodinas used in the present investigation were collected in 1963-1964 from crucian carp *Carassius carassius* from small ponds in Kortowo near Olsztyn. The precise data concerning host species and date of collection for each population are given in Table 1.

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The concept of a population, methods of preparation of the material, measuring of ciliates and statistical elaboration of the data have been presented in the Ist part of this paper (Kazubski 1991a).

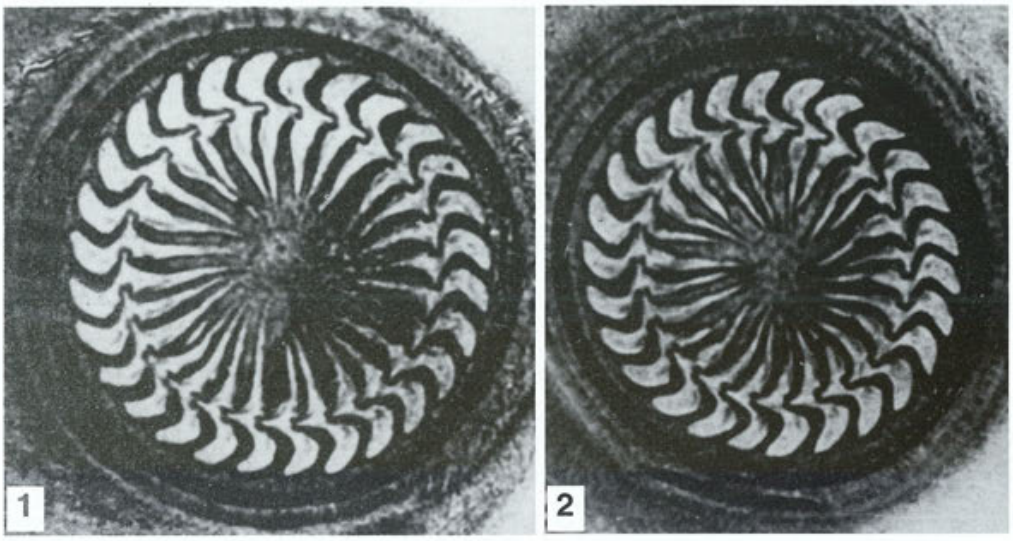
RESULTS

Trichodina pediculus from crucian carp has a typical pattern of the adhesive disc and denticulate ring; its denticles have semilunar blades and long, rather wide, dagger-like rays (Figs. 1 and 2).

First of all the material collected showed great differences in the level of infection of fishes at early spring and in summer. In March numerous trichodinas were observed on each of 9 fishes examined while only a few protozoans were noted on some (6 out of 23 examined) crucian carps examined in summer.

The results of measurements and counting are collected in Table 1 and visualized in Fig. 3. The table

comprises mean values for ciliates collected in summer (not fully formed specimens were not included), further the means and standard deviations for particular populations as well as means for the material collected in March and for the whole material are given. The diagram (Fig. 3) presents means and standard deviations concerning three characters in particular populations from March, in the whole material from March and in the material from summer.



Figs. 1 and 2. *Trichodina pediculus* from crucian carp, 1000x

The trichodinas collected in early spring were larger; the mean values counted for the whole groups were 9 to 12 per cent greater than the same values counted for summer specimens (Table 1).

Fairly great differences were observed also between means from particular populations of trichodinas in March. These differences amounted to 16.9 and 22.2 per cent for cell diameter and length of denticles, from 7.3 to 12.6 per cent for other dimensions of the adhesive disc and denticulate ring, and 7.7 per cent for the number of denticles. Occurrence of interpopulational variation was ascertained by the analysis of variance which showed moderately significant differences between means (Table 2). For the body diameter, adhesive disc diameter with border membrane, number of denticles and length of denticles the risk of error was lower then 1 per cent and variance ratio overlapped from 14 to 30 per cent the Fo value at proper number of degrees of freedom. The variation in the adhesive disc diameter without border membrane was statistically significant at the risk of error equal to 1.2 per cent, while denticulate ring diameter with the risk of error about 3.5 per cent.

DISCUSSION

The ciliates *T. pediculus* are fairly frequent parasites of fishes. In ponds in Kortowo (Olsztyn) they were found on crucian carps. In the same ponds and time they occurred also on hydras and tadpoles (Kazubski 1991a, b). It is worthy of mentioning that these ciliates occurred abundantly on crucian carps only in spring (in March) while in summer, when tadpoles and hydras were heavily infected, the crucian carps harboured only single specimens of these protozoans.

In *T. pediculus* from crucian carps clear diminishing of the cell dimensions has been observed in summer specimens in comparison with those collected in spring giving thus a proof on the occurrence of seasonal variation in this species of trichodina. Seasonal variation was also observed in *T. reticulata* occurring on crucian carps in the same water reservoirs (Kazubski 1982).

Statistical investigation made with the use of the one level analysis of variance has shown that in *T. pediculus* from crucian carps an interpopulational variation occurs at fairly low level, similarly as being observed on tadpoles (Kazubski 1991b).

A comparison of the ciliates *T. pediculus* from various host species, namely hydras, tadpoles and crucian carps has also been made. Comparison of mean dimensions of the cell and number of the denticles in most numerous, comparable groups of populations from these hosts has shown that the cell dimensions, dimensions of the adhesive disc and denticulate ring are much greater in ciliates from

Table 1

Mean values (M) and standard deviation (SD) of main characters of examined populations of *Trichodina pediculus* from crucian carp (*Carassius carassius* L.)
(n = sample numerosity)

Host	Date	No. of popul.	Diameter (μm)												Number of denticles			Length of denticles (μm)			Width of denticles (μm)
			body			adhesive disc with border membrane			adhesive disc			denticulate ring									
			M	SD	n	M	SD	n	M	SD	n	M	SD	n	M	SD	n	M	SD	n	
C. carassius																					
Kaczy Dołek	2.6.64	155	76.00	–	1	56.00	–	1	50.00	–	1	33.00	–	1	26	–	1	22.00	–	1	3.99
Kortowo	7.7.64	179	78.00	–	1	58.00	–	1	50.00	–	1	32.00	–	1	25	–	1	20.00	–	1	4.02
	„	180	80.00	–	1	60.00	–	1	50.00	–	1	34.00	–	1	27	–	1	20.00	–	1	3.96
	„	181	85.50	10.60	2	57.00	–	2	49.00	1.41	2	30.00	–	2	26	1.41	2	17.00	–	2	3.62
	total		81.00	6.86	5	57.60	1.52	5	49.60	0.89	5	31.80	1.79	5	26.00	1.00	5	19.2	2.17	5	3.84
C. carassius	16.3.65	297	86.27	11.37	26	59.35	4.05	26	52.77	3.74	26	34.79	2.44	26	27.36	0.99	25	19.50	1.68	26	3.99
Kaczy Dołek	31.3.65	298	94.03	12.10	30	62.87	4.77	30	55.10	4.26	30	36.63	2.66	30	28.17	1.34	30	19.31	1.65	29	4.09
Korowo	„	299	87.18	11.74	11	62.83	4.63	12	53.92	4.21	12	35.12	2.47	12	29.43	1.65	14	17.09	2.59	11	3.75
	„	300	99.67	13.37	6	66.83	4.62	6	57.50	5.17	6	37.33	3.84	6	28.33	2.07	6	19.83	1.83	6	4.14
	„	301	87.50	7.87	6	64.17	5.04	6	54.17	5.38	6	35.58	2.87	6	29.00	1.73	7	16.29	4.03	7	3.85
	„	302	85.29	9.47	28	61.89	4.31	28	53.96	3.96	28	34.80	2.90	28	27.93	1.36	28	19.46	2.28	28	3.91
	„	303	89.85	13.07	20	62.85	3.83	20	55.25	3.67	20	35.67	2.26	20	28.80	1.61	20	19.90	2.25	20	3.89
	„	304	88.33	7.12	30	64.20	4.05	30	56.53	3.48	30	35.87	2.31	30	28.67	1.54	30	19.30	1.21	30	3.93
	„	305	94.90	12.29	29	64.52	5.90	29	56.90	5.91	29	36.84	3.28	29	28.52	1.35	29	18.83	3.38	29	4.06
	total		89.96	11.47	186	62.87	4.84	187	55.10	4.49	187	35.80	2.78	187	28.35	1.51	189	19.12	2.39	186	3.97
Total, total			89.73	11.46	191	62.73	4.85	192	54.96	4.52	192	35.70	2.83	192	28.29	1.54	194	19.12	2.38	191	3.96

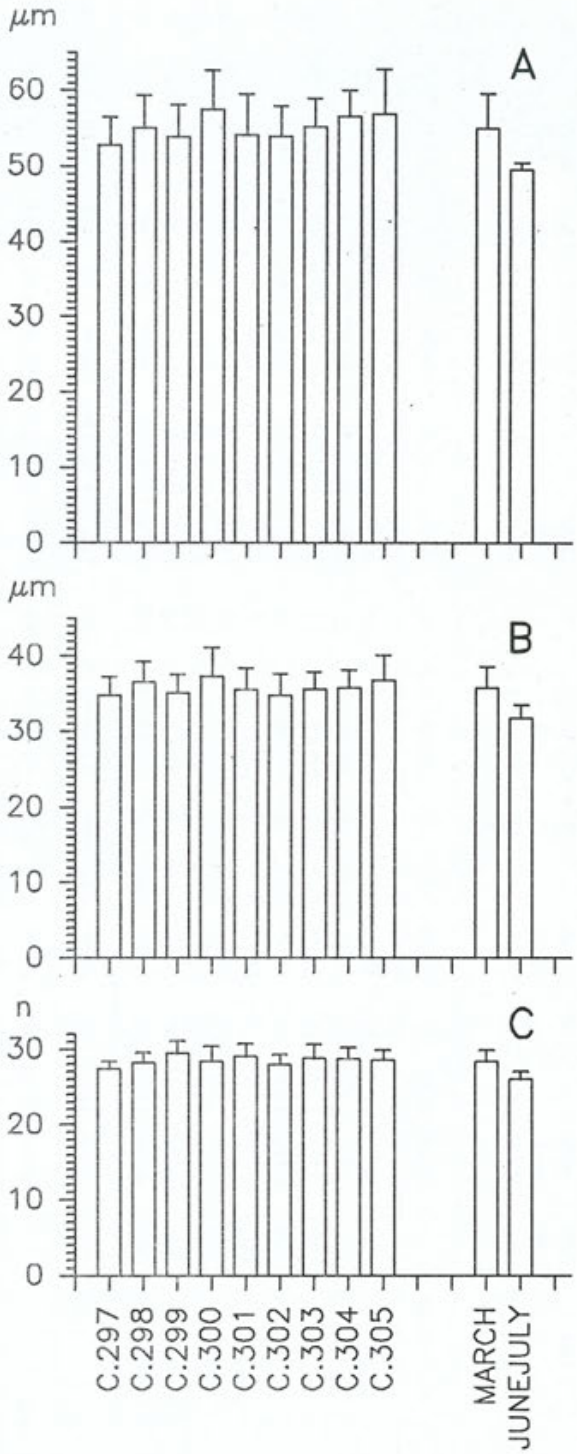


Fig. 3. Diagram of mean values and standard deviations of three characters: (A) diameter of adhesive disc, (B) diameter of denticulate ring, and (C) number of denticles in particular populations (on left) and local groups of populations (on right) of *Trichodina pediculus* from crucian carp

Table 2

One – level nested ANOVA table for six examined characters of 9 populations of *Trichodina pediculus* from crucian carp from Kortowo

Source of variation	Degree of freedom	Diameter of body		Diameter of adhesive disc with border membrane		Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles		Length of denticles		Critical value df	F. 01	F. 05
Among populations	9−1=8	F_0	3,033	1.1x	3.457	1.3x	2.581 ≈1.2%	2.151 ≈3.5%	3.506	1.3x	3.062	1.1x	(8,180)	2.663	2.016
Within populations	n−9	n=186			n=187		n=187	n=187	n=189		n=186				

Table 3

Mean values of six examined characters of *Trichodina pediculus* from hydras, tadpoles and crucian carp from Kortowo

	Diameter of								Number of denticles		Length of denticles	
	body		adhesive disc with border membrane		adhesive disc		denticulate ring					
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
Trichodinas from hydras populations Hv 26–29, Po 35–53	78.36	9.79 337	51.27	4.52 335	45.58	4.54 361	29.94	2.85 367	28.44	2.18 312	14.52	1.77 235
Trichodinas from tadpoles populations k 21–49	73.92	10.62 518	54.56	4.64 519	48.61	4.07 556	31.66	2.68 559	27.63	1.47 535	17.54	2.27 468
Trichodinas from crucian carp populations R 297-305	89.96	11.47 186	62.87	4.84 187	55.10	4.49 187	35.80	2.76 187	28.35	1.51 189	19.12	2.39 186

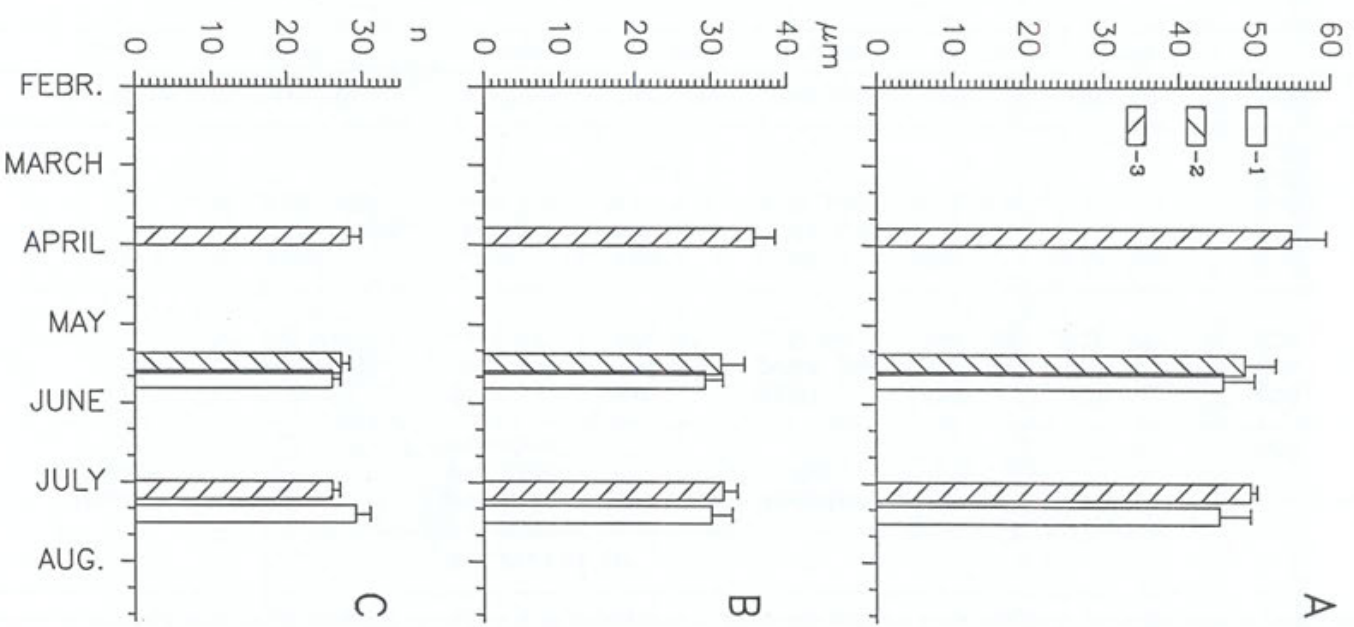


Fig. 4. Diagram of mean values and standard deviations of three characters: (A) diameter of adhesive disc, (B) diameter of denticulate ring, and (C) number of denticles of groups of *Trichodina pediculus* from (1) hydras, (2) tadpoles and (3) crucian carp in relation to seasons of the year

Table 4

Three – level nested ANOVA table for six examined characters of 49 populations of *Trichodina pediculus* from hydras, tadpoles and crucian carp from Kortowo

Source of variation	Degree of freedom	Diameter of body	Diameter of adhesive disc with border membrane	Diameter of adhesive disc	Diameter of denticulate ring	Number of denticles	Length of denticles	Critical value df	F. 01	F. 05
Among groups of populations from hydras, tadpoles and crucian carp	3−1=2	F_0 19.55	29.27	28.40	40.572	0.889	12.95	(2,6)	10.9	5.14
		F_s 12.91 1.2x	17.76 1.9x	18.06 1.9x	26.569 2.4x	0.487 ns	6.362 ≈2.7%	(2,7)	9.55	4.74
		df 5.82 ≈6	6.72 ≈7	6.71 ≈7	6.43 ≈6	7.66 ≈8	7.21 ≈7	(2,8)	8.65	4.46
Among local groups of populations	11−3=8	F_0 2.806	4.875	4.826	3.949	19.60	8.401 2.8x	(8,34)	3.09	2.23
		F_s 2.65 ≈2.5%	4.657 1.6	4.567 1.5x	3.776 1.3x	19.16 6.3x		(8,35)	3.07	2.22
		df 36.25 ≈36	36.60 ≈37	35.42 ≈35	34.35 ≈34	36.43 ≈36		(8,36)	3.05	2.21
								(8,37)	3.04	2.20
								(8,38)	3.02	2.19
Among population	49−11=38	F_0 3.335 2.0x	3.169 1.9x	2.492 1.5x	1.848 1.1x	2.047 1.2x	3.080 1.9x	(35,1000)	1.66	1.44
Within population	n−49	n=1041	n=1041	n=1104	n=1113	n=1036	n=889			

crucian carps than from other hosts (Table 3). Only the number of denticles rests stable. The nested analysis of variance performed on the material from these three groups of hosts from Kortowo, with respect paid to variation in local groups, has shown differences between means at fairly low level. The differences concerning the number of denticles were statistically insignificant (Table 4).

More precise analysis of these results, taking into account the date of collection of the material (Fig. 4), seems to suggest that these ciliates display the seasonal variation rather than the variation influenced by the host species. Populations from crucian carps, used for this consideration, were collected in March, while those from hydras and tadpoles in June and July. Simultaneously, mean values of the characters examined of *T. pediculus* from crucian carps collected in summer virtually did not differ from mean values of these characters in trichodinas from other host species collected at the same time.

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ACTA
PROTOZOOLOGICA

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Amphorocephalus yoshidaensis sp.n. *(Apicomplexa, Gregarinasina, Actinocephalidae)* from the Chilopod *Bothropolys asperatus* in Japan

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Summary. The septate gregarine *Amphorocephalus yoshidaensis (Apicomplexa, Gregarinasina, Actinocephalidae)* is described from the intestine of the chilopod *Bothropolys asperatus* in Japan. Its gamonts average 262 by 86 μm; their protomerites average 61 μm long and 73 μm wide, and their deutomerites average 201 μm long and 86 μm wide. Its gametocysts are spherical and average 102 μm in diameter; their wall is 2-layered, the outer layer gelatinous and 10 μm thick, and the inner layer transparent and 1 μm thick. Each gametocyst contains many fusiform oocysts 7-8 x 4-5 μm. Dehiscence is by simple rupture.

Key words. *Amphorocephalus yoshidaensis* sp.n., gregarine, parasites, chilopod *Bothropolys asperatus*

INTRODUCTION

Approximately 2,800 species of *Chilopoda* (centipedes) have been reported from various parts of the world, but gregarines have been found in only 51 species. The 43 species of gregarines, belonging to 15 genera and 5 families, have been reported by Crawley (1903), Ellis (1913), Geus (1969), Hoshide (1958), Levine (1979) and Schneider (1875). They principally belong to *Dactylophoridae* (26 species) and *Actinocephalidae* (13 species) as listed by Levine (1988). The new species reported here was isolated from the family *Lithobiidae* and provides additional evidence of the widespread occurrence of gregarine associations with this group of centipedes.

MATERIAL AND METHODS

The host chilopod, *Bothropolys asperatus* (L. Koch), was collected in the woods on the bank of the Fushino River at Yoshida, Yamaguchi-shi, near the campus of Yamaguchi University, Japan. The chilopods were kept in small Petri dishes lined with wet filter paper on their bottoms. Gametocysts were collected in the feces of the hosts. They were kept in the moist chamber, 2 or 3 days until they dehisced their oocysts.

To observe the gamonts, the head and tail of the chilopod were cut off in Ringer's solution with fine needles. Most observations were made with living specimens, but some were fixed in Bouin's fluid and stained with Delafield's hematoxylin or fixed in 5% formaldehyde solution and stained with methyl green. The all measurements shown bellow were done on living specimens in Ringer's solution.

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RESULTS

The gregarine found in *B. asperatus* was found to belong to the genus *Amphorocephalus* Ellis, 1913 but to be a new species. Its gamonts, cephalines, gametocysts and oocysts are described below.

G a m o n t s (Figs. 1A, 2B): These are solitary and elongate ovoid. The largest individual seen was 302 μm long and 99 μm wide. The gamonts averaged 262 μm in total length, with a protomerite 61 μm long and 73 μm wide, and a deutomerite 201 μm long and 86 μm wide.

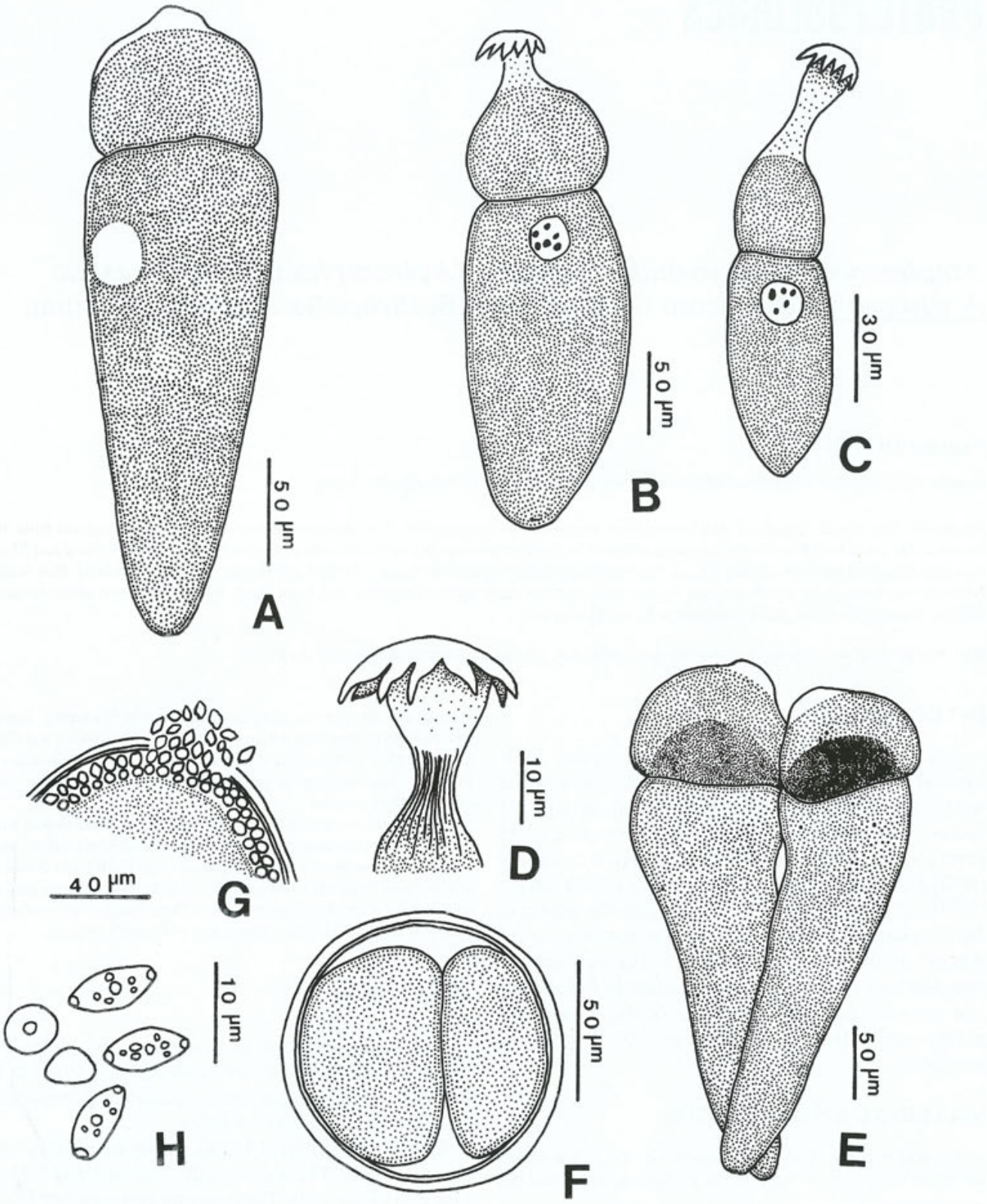

Fig. 1. *Amphorocephalus yoshidaensis* sp.n. A - Mature gamont, B,C - Cephalines with epimerite, D - Epimerite (enlarged), E - Associated gamonts, F - Gametocyst, G - Oocysts being dehisced from a gametocyst by simple rupture, H - Oocysts

The protomerite was dome-shaped or subglobular, with a small conical papilla at its apex. It was slightly wider than high, being widest a little above the septum. The deutomerite was elongate ovoid or somewhat cylindrical, widened sharply from the septum, being widest just below the shoulder and then tapering down to a sharply pointed or sometimes broadly rounded posterior end. The septum between the protomerite and deutomerite was conspicuous and formed a deep constriction. The nucleus was ellipsoidal or spherical, but its outline was not clear because of the dense endoplasm. The nucleus was generally in the anterior

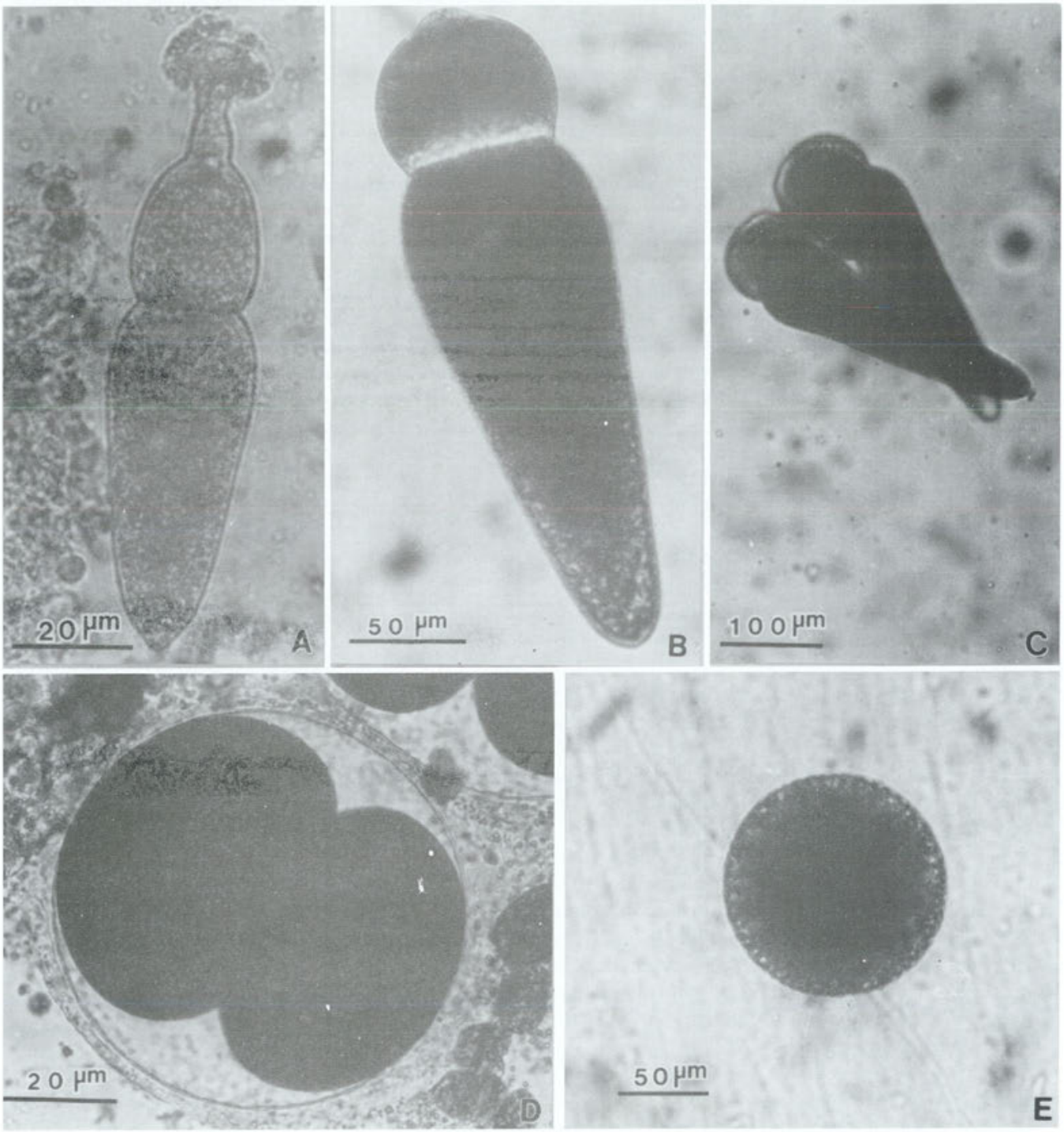

Fig. 2. *Amphorocephalus yoshidaensis* sp.n. A - A cephaline with an epimerite, B - Mature gamont, C - Two gamonts beginning head-to-head association, D - Young gametocyst containing two individuals, E - Mature gametocyst containing many oocysts

half of the deutomerite. It contained 8-10 and sometimes more nucleoli. Its endoplasm was light brown, and contained both large and fine granules. It was denser in the deutomerite and posterior half of the protomerite than in the anterior half of the protomerite, and was almost transparent and formed a small cone at the top of the body. The ectoplasm was fairly thick. The gamont moved slowly forward by sliding and the anterior part of the body was sometimes bent.

Cephalines (Figs. 1B, C, 2A): The cephalines were ovoid or elongate ovoid. They gradually became larger as they grew older. The small cephalines were lighter in color than the mature gamonts. They had fine, scanty granules in both the protomerite and deutomerite. The nucleus was spherical. The epimerite consisted of a crown borne on a short, cylindrical stalk, which rested on the apex of the conical projection of the protomerite and had several fine, longitudinal striations on its surface. In some small cephalines the stalk was as much as twice the height of the protomerite. The crown of the protomerite was globular and bore 8-10 backward-pointing, rigid spines on its anterior margin.

Gametocysts (Figs. 1F, 2D, E): The two gamonts came in the association by head to head and make the gametocysts (Fig. 1E). The gametocysts were spherical, with an external diameter of 102 μm; their contents were 82 μm in diameter. The gametocyst wall was composed of two layers, the outer one gelatinous and 10 μm thick, and the inner one transparent and 1 μm thick. Dehiscence was by simple rupture (Fig. 1G).

Oocysts (Fig. 1H): The oocysts were 7-8 x 4-5 μm. They were generally fusiform and almost circular in section.

This species is named *Amphorocephalus yoshidaensis* sp.n. The species name is based on the name of the place where the host chilopods were found.

Type specimens. Gamonts fixed in Bouin's fluid, stained Delafield's hematoxylin and mounted on the slides. The slide number of the holotype is 87001 and the slides number of paratype are 87002-87012. The holotype and paratype materials are at present deposited at the Biological Institute, Faculty of Educatioan, Yamaguchi University,to be submitted later to the National Science Museum, Tokyo.

DISCUSSION

Because of the characteristic shape of its epimerite, this species belongs to the genus *Amphorocephalus* (Ellis 1913).

Six species of *Amphorocephalus*, all from the intestine of chilopods, have been named previously: *A. actinotus* (Leidy, 1889) Ellis, 1913 from *Scolopocryptops* spp. in North Carolina and Pennsylvania, USA; *A. amphorellus* Ellis, 1913 from *Scolopendra* heros in Colorado, USA; *A. aratoensis* (Hukui, 1951) Levine, 1980 from *Cryptops japonicus* in Japan; *A. bouruiensis* (Hukui, 1952a) Levine, 1980 from *Otocryptops rubiginosus* in Japan; *A. ozakii* (Hukui, 1952b) Levine, 1980 from *Otocryptops rubiginosus* in Japan; and *A. scolopendrae* (Crawley, 1903) Levine, 1980 from *Scolopendra woodi* in North Carolina.

The present species is more like *A. bouruiensis* than any of the other species. However, it differs from that species in that (1) it is smaller (mean size 262 × 86 μm in contrast to *A. bouruiensis*, which is 875 × 117 μm), (2) its epimerite bears 8-10 backward-pointing, rigid spines at its anterior margin, whereas the epimerite of *A. bouruiensis* has several irregular-formed, finger-like processes at its posterior part, (3) its gametocysts are two to three times smaller than those of *A. bouruiensis*, and (4) its oocysts are fusiform, whereas those of *A. bouruiensis* are spherical. While differences in size may be simply a result of the difference in host, differences in oocyst and epimerite structure are not. Hence the establishment of a new species is justified.

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ACTA PROTOZOOLOGICA

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Stenophora tamulini sp. n. *(Apicomplexa: Cephalina)* from the Centipede, *Tamulinus ceylanicus* (Attems) of Kerala, India

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Summary. The paper deals with the description of morphology and life-history of a new species of cephaline gregarine, *Stenophora tamulini* sp. n. infecting the centipede, *Tamulinus ceylanicus* (Attems), collected from Ezhimala in the Cannanore district of Kerala. The sporadin has the following ratios: PL : TL = 1 : 9.1; PW : DW = 1 : 1.2.

Key words. *Stenophora tamulini* sp.n., gregarine, parasites, centipedes, *Tamulinus ceylanicus.*

INTRODUCTION

Five species of cephaline gregarines belonging to 4 genera, *Stenophora* Labbè, 1899, *Grebneckiella* Bhatia, 1938, *Mecistophora* Ganapati and Narasimhamurti, 1960 and *Chilogregarina* Levine, 1979 have been reported from Indian Centipedes (Mitra and Chakravarty 1937, Chakravarty 1939, Misra 1942, Ganapati and Narasimhamurti 1960, Sarkar 1987 and Prema and Janardanan 1987).

While examining the centipedes of Kerala for their gregarine parasites, we came across a species of *Stenophora* in the intestine of *Tamulinus ceylanicus* collected from Ezhimala in the Cannanore district of Kerala. It is reported here as *Stenophora tamulini* sp. n.

MATERIAL AND METHODS

The centipedes, *Tamulinus ceylanicus* (Attems) collected from Ezhimala in the Cannanore district of Kerala were brought alive to the laboratory and examined for their gregarines. Trophozoites and sporadins recovered from midgut and gametocysts from hindgut and/or faecal pellets were studied following Prema and Janardanan (1987). Sporozoites were released by exposing the spores to host's midgut fluid and observed under a phase-contrast microscope. Illustrations were made with the aid of camera lucida.

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

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RESULTS

Stenophora tamulini sp. n.

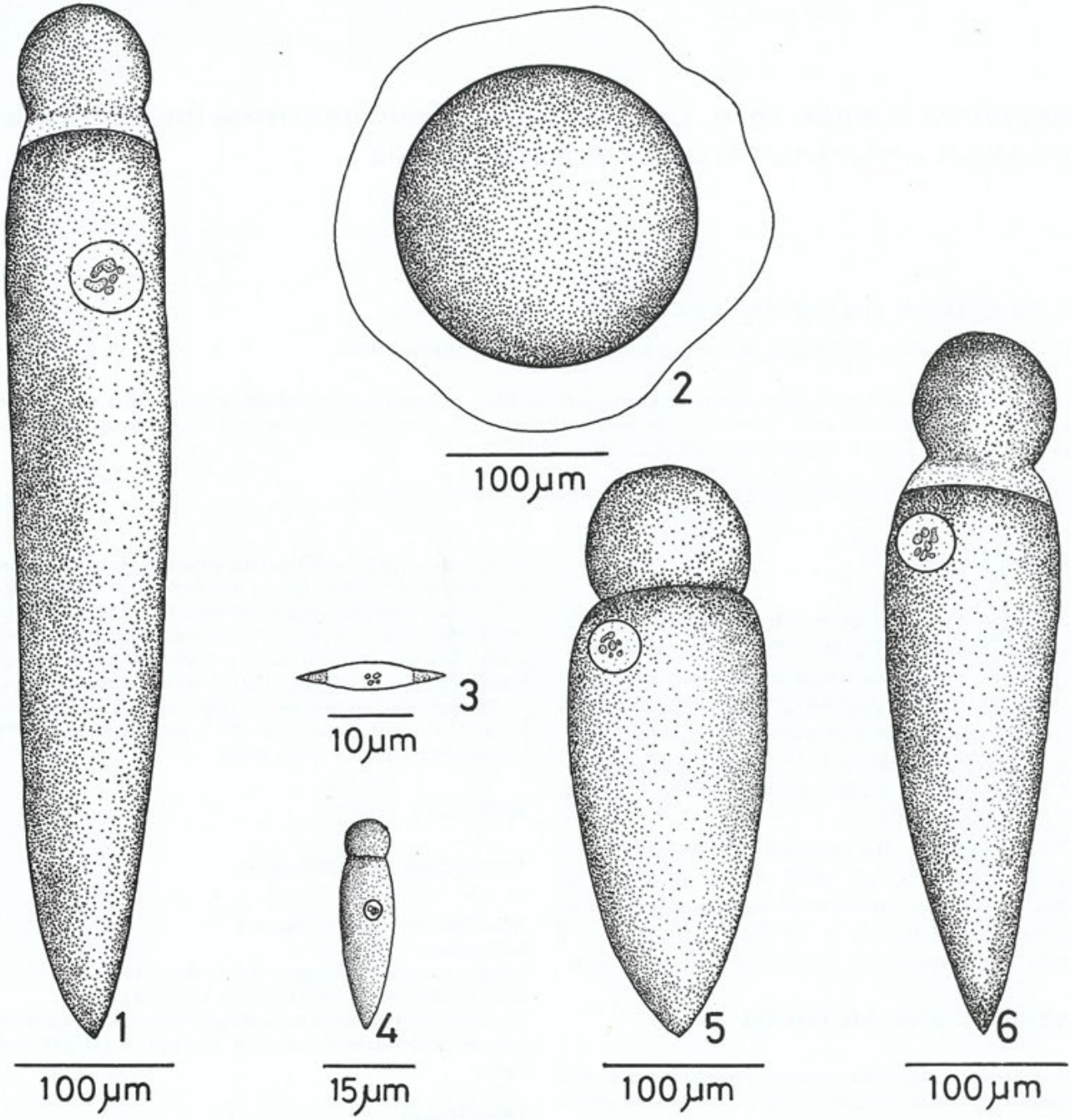
Host: *Tamulinus ceylanicus* (Attems)
Site: Intestine
Locality: Ezhimala, Cannanore district, Kerala (India)
Date of collection: June to October of 1988 and 1989
Holotype: To be deposited in the parasite collections, Parasitology Laboratory, Department of Zoology, University of Calicut, Kerala (India)

Description

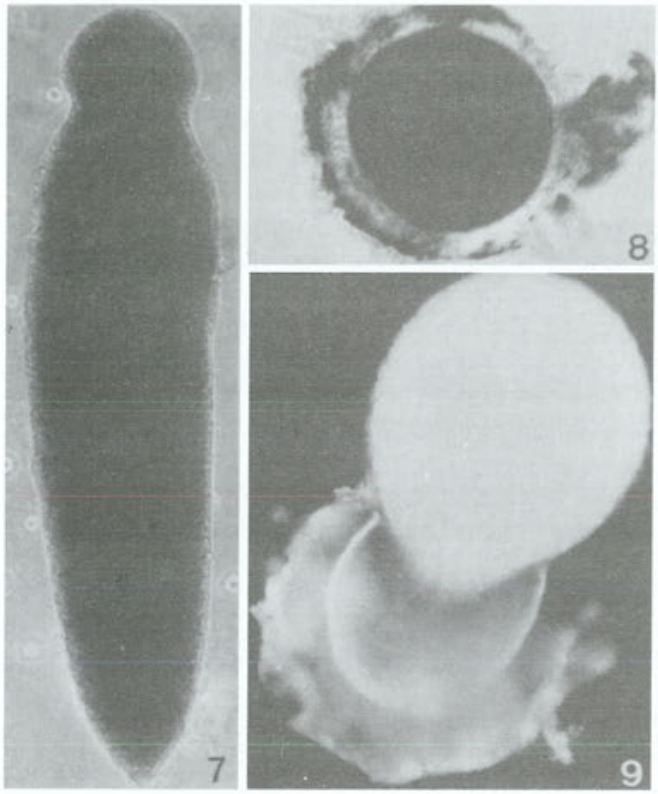
S p o r a d i n s (Figs. 1, 7): Sporadins solitary, milky-white, elongate, cylindrical, gradually tapering to

the posterior and ending in a pointed caudad. Protomerite dome-shaped, with a constriction behind the anterior two-third; apex without papilla and pore. Protomerite epicyte uniformly thick, striated, striations continuous with those on deutomerite; endocyte granular, a granule free area present just above the septum. Septum thin, circular, slightly convex toward anterior; constriction at septum conspicuous.

Deutomerite elongate, cylindrical, gradually tapering to a slightly pointed caudad; epicyte uniformly



Figs. 1-6. *Stenophora tamulini* sp. n. from *Tamulinus ceylanicus* (Attems). 1 - Sporadin; 2 -Gametocyst; 3 - Spore; 4-6 - Lumen trophozoites



Figs. 7-9. Development stages of *Stenophora tamulini* sp. n. 7 - Sporadin; 8 - Gametocyst; 9 - Dehiscence of gametocyst

thick, longitudinally striated; endocyte granular.

Nucleus spherical, feebly visible in fresh sporadins, variable in position. Endosomes irregular, two to six in number, deep-staining with hematoxylin. Nucleus in a sporadin of 706 by 92 μm measured 42 μm in diameter.

Measurements of sporadins in micrometers (with mean in parentheses) are noted below:

DL = 346 - 617 (544); DW = 33 - 108 (88);
PL = 52- 88 (67); PW =46-95 (74);
TL = 427 - 706 (611).

Ratios:

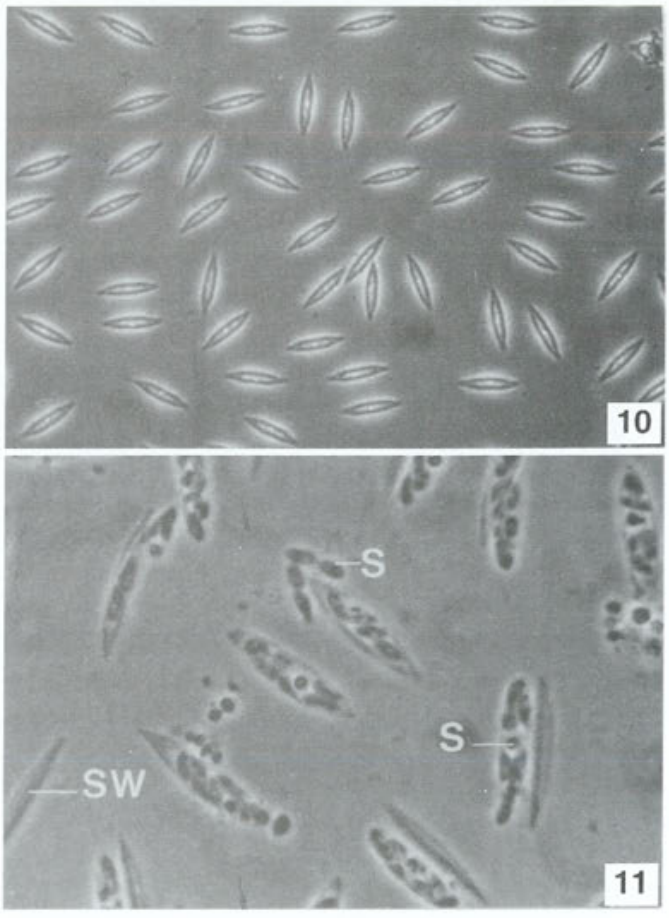
PL : TL = 1 : 5.3 - 12.4 (1 : 9.1);
PW : DW = 1 : 1.1 - 1.3(1 : 1.2).

G a m e t o c y s t s (Figs. 2, 8): Gametocysts spherical, opaque, milky-white. Cyst wall hyaline, uneven, 27-52 μm (33 μm) thick. Line of association clearly visible in fresh gametocysts. Gametocysts measured 182-223 μm (206 μm).

S p o r e s (Figs. 3, 10): Spores spindle-shaped, 18 by 3.8 μm.

B i o l o g y: Gametocysts maintained in moist chamber at room temperature (28°C - 30°C) developed spores in 96 h. At about 6 h before dehiscence, a clear zone without inclusions differentiated at the periphery below the cyst wall. Mature spores appeared to aggregate at the central dense area of the cyst. After 96 h of incubation, the whole spore mass came out through a rupture in the cyst wall (Fig. 9). On exposure to moisture the spore mass disintegrated liberating the spores.

The liberation of sporozoites was induced by placing fresh spores in the host's midgut fluid, which activated the sporozoites. The sporozoites started wriggling inside the spores after 16 min of exposure to midgut fluid; after 18 min, the spore wall split up longitudinally into two halves and liberated the sporozoites (Fig. 11). The centipedes seem to pick up



Figs. 10-11. Spores of *Stenophora tamulini* sp.n. 10 - Spores; 11 - Liberation of sporozoites. S - Sporozoites, SW - Spore wall

infection by ingesting viable spores along with food materials. The sporozoites are released in midgut, get attached to the midgut epithelium and develop into trophozoites. Intracellular development stages were not observed. The smallest observed trophozoite (Fig. 4) measured 34.7 by 9.9 μm, had a hemispherical, 6.6 μm long protomerite and 28 μm long deutomerite containing a spherical nucleus. The protomerite remained hemispherical (Figs. 5, 12) until the trophozoites

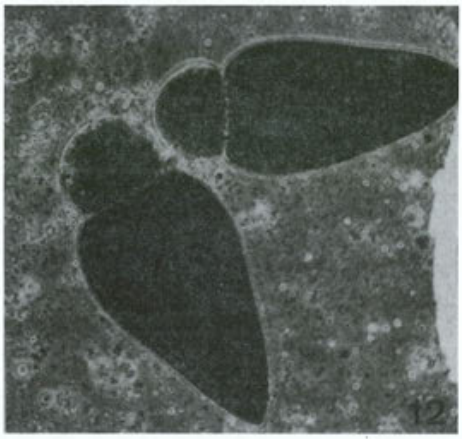


Fig. 12. Trophozoites of *Stenophora tamulini* sp.n

reached a length of 365 μm. With further growth the protomerite became dome-like with a constriction behind the anterior two-third (Fig. 12). With further growth and accumulation of reserve food in the cytoplasm, the trophozoites developed into sporadins.

DISCUSSION

The present gregarine from *Tamulinus ceylanicus* has solitary sporadins; its gametocysts dehisce by simple rupture. Based on these characters the gregarine is included under the genus *Stenophora* Labbè, 1899.

The present gregarine needs comparison with the only species of *Stenophora, S. shyamaprasadi* Chakravarty, 1939, reported from a centipede host. It significantly differs from *S. shyamaprasadi* in morphology and morphometry of sporadins, in the absence of epimerite, and in having spherical nuclei with 2-6 irregular endosomes and spores which are spindle-shaped. The gregarine is, therefore, considered a new species and is reported here as *Stenophora tamulini* sp. n., after its host. This forms the second species of *Stenophora* from a centipede host.

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Gregarines (*Apicomplexa: Eugregarinida*) from Cave and Terrestrial Arthropods in Bulgaria

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Summary. Seven eugregarine species are reported from *Insecta, Chilopoda* and *Diplopoda* collected from terrestrial and cave habitats. Three eugregarines are described as new species, namely *Gregarina troglophili* n.sp. from *Troglophilus neglectus* Krauss, *Stenophora gervaisiae* n.sp. from *Gervaisia (Trachysphaera) costata* Waga, and *Stenophora serboiuli* n.sp. from *Serboiulus spelaeophilus* Gulicka. *Bulgorosoma meridionale* Tabacaru was established as a new host for *Stenophora bulgarosomae* Golemansky and *Pachyiulus hungaricus* (Karsch) as a new host for *Stenophora juli* (Frantzius).

Key words. *Eugregarinida*, gregarines, parasites, cave and terrestrial arthropods.

INTRODUCTION

Fauna of eugregarines of arthropods in Bulgaria is not well known. Corbel (1964) was first who recorded and described two new eugregarine species from *Orthoptera*. In a series of papers Golemansky (1973), Golemansky and Taschev (1973), Taschev and Golemansky (1973) reported five eugregarines from some cave inhabiting arthropods (troglobionts and troglophilous species); four eugregarine species were described as new species to science.

In the last years Golemansky and Duhlinska (1982), and Lipa, Golemansky and Duhlinska (1991) described, as new species to science or newly recorded for Bulgaria, several eugregarines from *Insecta* and *Crustacea*. As the result of the above mentioned studies the eugregarine fauna of Bulgaria consists of 16 species, among which 6 species were described as new taxa.

In this paper we present additional data on eugregarine fauna of *Insecta, Chilopoda*, and *Diplopoda* in Bulgaria.

MATERIAL AND METHODS

Arthropod hosts for this study were collected in various biotopes during the period from 1973 to 1990. Special attention was given to the troglobionts or to troglophilous species of arthropods which part or the whole life spend in the mountain caves. The host arthropods were brought alive to the laboratory, dissected, and the recorded gregarines were measured in Ringer's solution, drawn or photographed using the light microscope Nu-2 Zeiss, Jena. The material for nucleus and cytoplasm studies was fixed in Schaudinn's fluid and stained with Ehrlich's hematoxylin; acetocarmin was used for rapid coloration of the nucleus.

The holotype slides of the newly described species are deposited in the collection of the Institute of Zoology, Bulgarian Academy of Sciences in Sofia.

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

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Table 1

List of eugregarines with their hosts recorded during this study in Bulgaria

Host *Arthropods*	*Eugregarines*
Orthoptera	
Troglophilus neglectus Krauss	*Gregarina troglophili* sp.n.
Dilopoda	
Bulgarosoma meridionale Tabacaru	*Stenophora bulgarosomae* Golemansky
Gervaisia (Trachysphaera) costata Waga	*Stenophora gervaisiae* sp.n.
Pachyiulus hungaricus (Karsch)	*Stenophora juli* (Frantzius)
Polydesmus complanatus (L.)	*Amphoroides polydesmi* (Leger)
Serboiulus speleophilus Gulicka	*Stenophora serboiuli* sp.n.
Chilopoda	
Scolopendra cingulata L.	*Grebneckiella gracilis* (Grebnecki)

Table 2

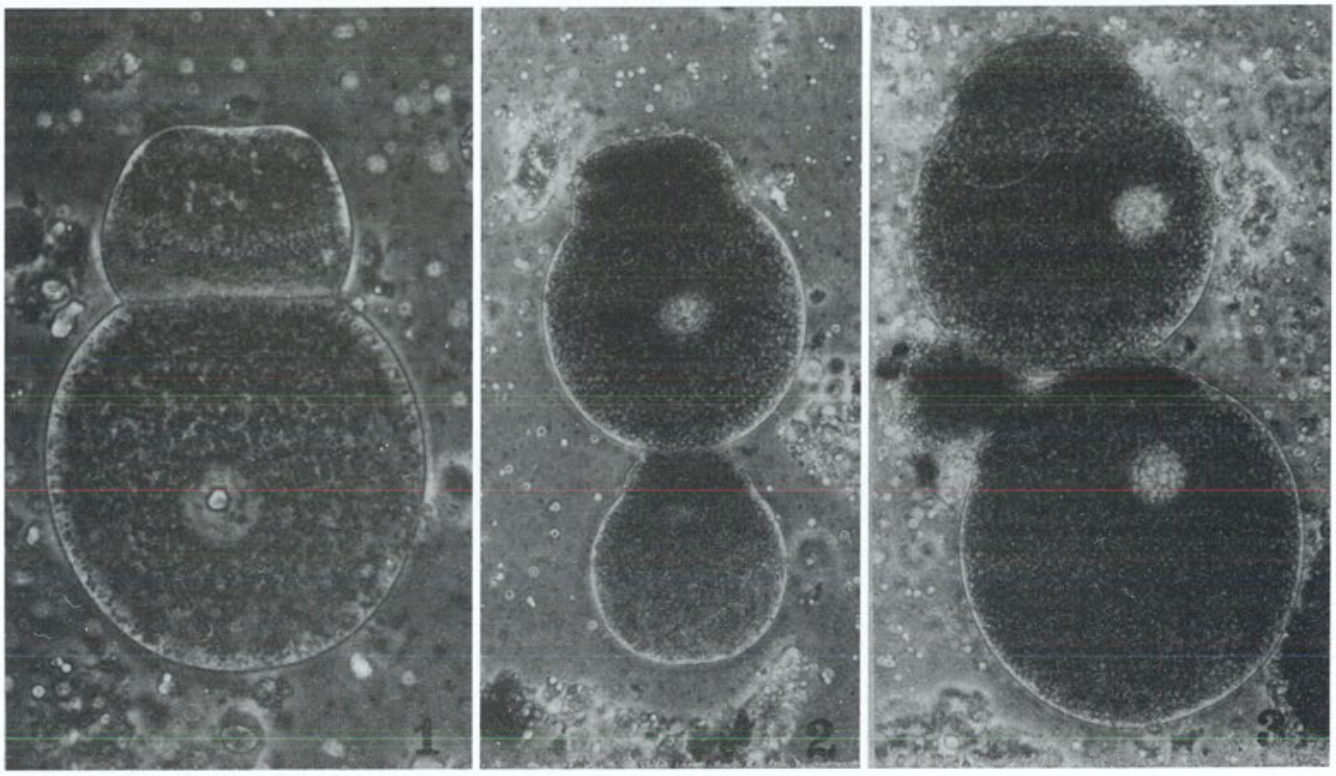
Measurements of cephalonts and sporonts of *Gregarina troglophili* sp.n.

TL (μm)	LP (μm)	LD (μm)	WP (μm)	WD (μm)	LP:TL	WP:WD	LP:WP
Cephalonts:							
104	30	74	50	81	1:3.41	1:1.60	1:1.65
139	38	101	74	105	1:3.65	1:1.42	1:1.93
139	25	114	76	114	1:5.50	1:1.50	1:3.00
Sporonts:							
199	38	161	107	160	1:5.22	1:1.50	1:2.80
208	44	164	120	220	1:4.72	1:1.83	1:2.72
213	38	175	136	203	1:5.59	1:1.49	1:3.56
236	52	184	84	120	1:4.53	1:1.42	1:1.61
250	45	205	120	200	1:5.55	1:1.60	1:2.66

Table 3

Measurements of cephalonts and sporonts of *Amphoroides polydesmi* (Leger)

TL (μm)	LP (μm)	LD (μm)	WP (μm)	WD (μm)	LP:TL	WP:WD	LP:WP
Cephalonts:							
132	8	124	20	40	1:16.50	1:2.00	1:2.50
140	12	128	24	52	1:11.66	1:2.16	1:2.00
160	12	148	40	104	1:13.33	1:2.60	1:3.33
Sporonts:							
260	20	240	37	96	1:13.00	1:2.59	1:1.85
308	20	288	40	120	1:15.40	1:3.00	1:2.00



Figs. 1-3 *Gregarina troglophili* sp.n.: 1 – cephalont (x400), 2-3 – sporonts in syzygies (x200)

RESULTS

Altogether seven eugregarine species were recorded in arthropods belonging to *Orthoptera, Chilopoda* and *Diplopoda* (Table 1).

Gregarina troglophili sp.n. (Figs. 1-3)

Host: *Troglophilus neglectus* Krauss *(Orthoptera, Gryllidae)*.
Locality: Caves "Karnata" and "Imamova Dupka" in Rhodopa Mountains: 18.X.1973 (leg. V. Golemmnsky), 31.I.1980 (leg. D. Raichev).

Morphology

C e p h a l o n t s: Young cephalonts were not observed. Mature cephalonts observed varied in length from 104 to 140 μm (Table 2). Epimerite globular or subspherical, 20-24 μm in diameter. Protomerite hemispherical, well separated from the deutomerite by constriction (Fig.1). Deutomerite oval, with globular nucleus containing mostly one karyosome. Paralel cuticular striations were noticed.

S p o r o n t s: Sporonts in syzygies (Figs. 2,3) and their body length varied from 199 to 250 μm (Table 2). Protomerite large, lunar shaped with non-visible septum and constriction especially in satellite. Endocyte of protomerite and deutomerite non-translucent, dark and contains large paraglycogen granules.

In general the body size of primite and satellite in syzygies is similar but in some syzygies the satellite is much smaller (Fig. 2).

Cysts were not seen.

Taxonomy

Only three species from *Gregarina* genus are known from *Gryllidae*, namely *Gregarina oviceps* Diesing (1959), *G. macrocephala* (Schneider) Labbe (1899) and *G. grylli* Corbel (1968), parasitizing in *Gryllus, Acheta* and *Nemobius* genera.

This is the first record of gregarine infection in *Troglophilus neglectus* which is a cave inhibiting insect with very limited contacts with other gryllids from which it could harbor the parasitic gregarines.

Considering that the gregarine recorded in *T. neg-*

lectus differs from previously known gregarines by the globular form of cephalonts and sporonts, the type of epimerite and the small body size we consider that this is a new species for which we propose the name *Gregarina troglophili* sp.n.

Amphoroides polydesmi (Leger) (Figs. 4-7)

Host: *Polydesmus complanatus* (L.) *(Diplopoda: Polydesmidae).*
Locality: Gorna Banya (near Sofia): 29.III.1990 (leg. V. Golemansky)

Morphology

C e p h a l o n t s: The body elongated, narrowing in the front part, up to 160 μm long (Table 3). Endocyte translucent, granulated. Protomerite hemispherical, separated from deutomerite by well visible septa. Nucleus globular, about 15 μm in diameter. The epicite with longitudinal striations.

S p o r o n t s: The largest observed sporons measured 308 μm (Table 3). The end of the deutomerite of sporont is rounded but in some individuals a well marked caudal region was seen (Fig. 7).

Taxonomy

The morphology of cephalonts and sporonts in the studied material corresponds to the original description of *Amphoroides polydesmi*. Geus (1969) evidently observed only cephalonts with the length of 172 μm.

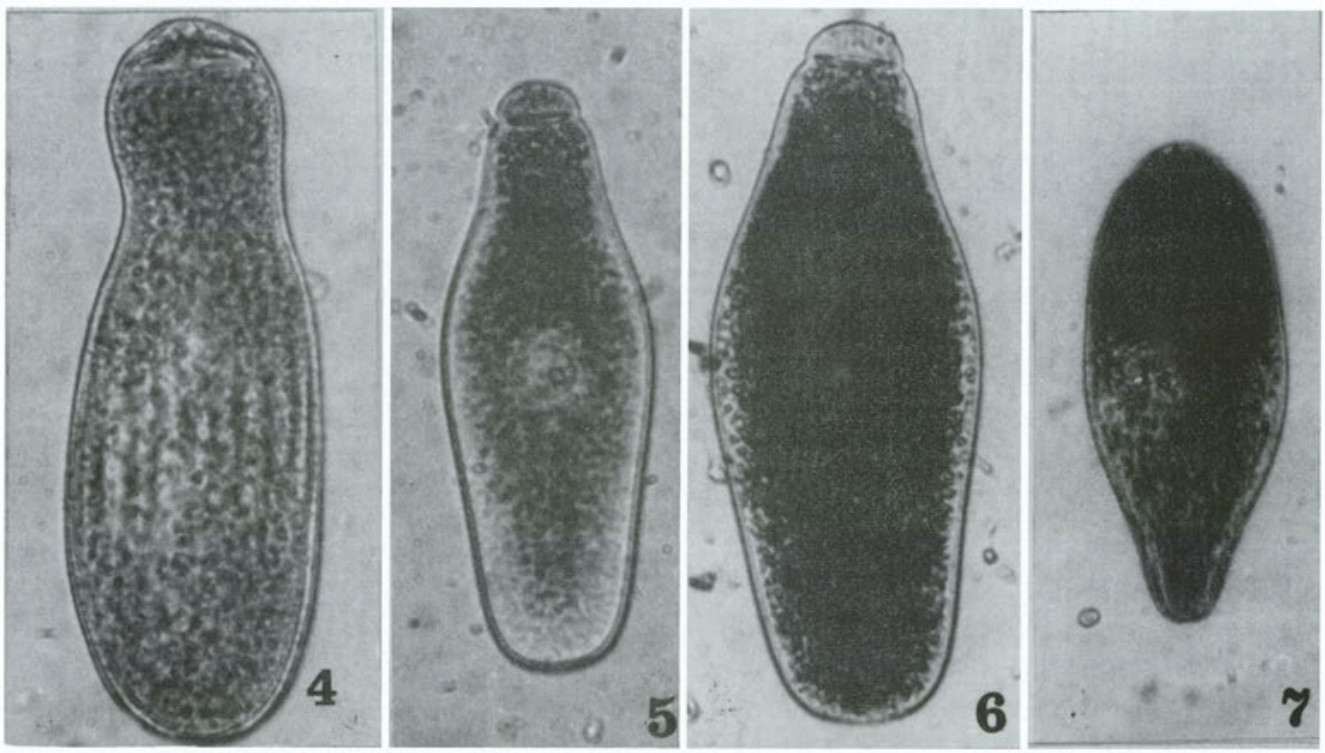
Stenophora gervaisiae sp.n. (Figs. 8-11)

Host: *Gervaisia (Trachysphaera) costata* Waga *(Diplopoda, Gervaisiidae).*
Locality: Cave "Er Kjupria" (near Mostovo) in Rhodopa mountains. 23.X.1973 (leg. V. Golemansky)

Morphology

C e p h a l o n t s: Young cephalonts (Figs. 8,9) had oval body with distinct protomerite, and measured 30-46 μ. Deutomerite oval in young cephalonts becomes elongated in older ones (Fig. 9) which measured up to 213 μm (Table 4). Endocyte transparent without paraglycogen granules. Nucleus round with one karyosome.

S p o r o n t s: Elongated, having deutomerites frequently narrowed in their caudal part. Endocyte dense, with paraglycogen granules more distinct in protomerite. The largest sporonts were up to 348 μm long (Table 4). Nucleus oval up to 25 μm in diameter, contains one karyosome.

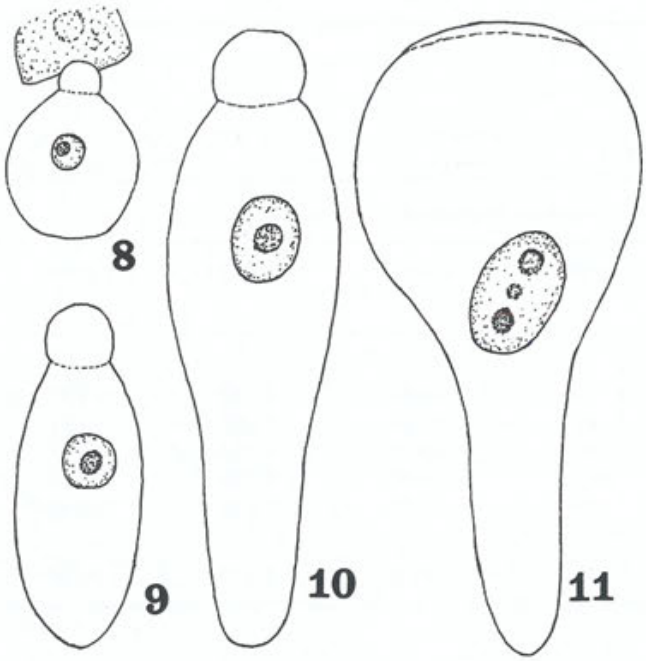


Figs. 4-7 *Amphoroides polydesmi* (Leger): 4 - young cephalont (×400), 5-7 - sporonts (×700)

Table 4

Measurements of cephalonts and sporonts of *Stenophora gervaisiae* sp.n.

TL (μm)	LP (μm)	LD (μm)	WP (μm)	WD (μm)	LP:TL	WP:WD	LP:WP
Cephalonts:							
30	5	25	5	25	1:6.08	1:5.08	1:1.00
46	8	38	10	18	1:6.00	1:1.74	1:1.34
138	13	125	17	30	1:10.84	1:1.79	1:1.33
208	20	188	28	53	1:10.26	1:1.89	1:1.37
213	18	195	25	53	1:11.83	1:2.08	1:1.41
Sporonts:							
266	18	248	28	53	1:14.93	1:1.91	1:1.56
271	15	256	28	76	1:17.84	1:2.71	1:1.84
348	13	336	33	61	1:27.45	1:1.84	1:2.59



Figs. 8-11 *Stenophora gervaisiae* sp.n.: 8-9 - young cephalonts (×1000), 10 - elongated sporont (×400), 11 - gamont (×400)

The soronts (gamonts) ready for sexual reproduction have very flat protomerite and their deutomerite becomes round with dostinct caudal part (Fig. 11).

Taxonomy

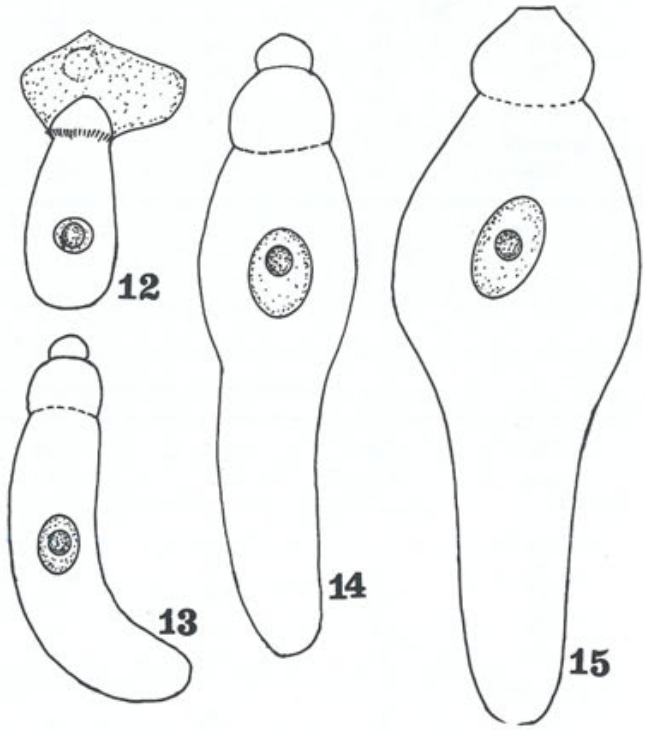
This is the first record of eugregarine infection in *Gervesia costata*. The morphology of protomerite and deutomerite, as well as ratio LP:TL, differ significantly the recorded species from other *Stenophora* species known from diplopods. Therefore we consider it is a new species and the name *Stenophora gervaisiae* sp.n. is proposed for it.

Stenophora serboiuli sp.n. (Figs. 12-15)

Host: *Serboiulus speleophilus* Gulicka, 1967 *(Diplopoda, Julidae)*.
Locality: Cave "Vodna Pe'" near Vidin: 13.VI.1973 (leg. P. Baron).

Morphology

Cephalonts: The youngest cephalonts are elongated and cylindral up to 50 μm in length and are



Figs. 12-15 *Stenophora serboiuli* sp.n.: 12-14 - cephalonts at various stages of maturation (×1000), 15 - sporont (×400)

Table 5

Measurements of cephalonts and sporonts of *Stenophora serboiuli* sp.n.

TL (μm)	LP (μm)	LD (μm)	WP (μm)	WD (μm)	LP:TL	WP:WD	LP:WP
Cephalonts:							
37	7	30	8	15	1:5.28	1:1.93	1:1.14
50	8	42	8	15	1:6.52	1:1.87	1:1.05
106	10	96	10	15	1:10.60	1:1.54	1:1.00
162	12	150	15	22	1:13.00	1:1.44	1:1.21
186	18	168	20	30	1:10.33	1:1.50	1:1.11
195	15	180	18	25	1:13.00	1:1.38	1:1.20
Sporonts:							
305	20	285	30	89	1:15.03	1:2.91	1:1.50
348	28	320	38	100	1:12.42	1:2.63	1:1.35
397	23	374	33	83	1:17.40	1:2.51	1:1.44
508	23	485	38	128	1:22.08	1:3.36	1:1.65

Table 6

Measurements of cephalonts and sporonts of *Stenophora bulgarosomae* Golemansky

TL (μm)	LP (μm)	LD (μm)	WP (μm)	WD (μm)	LP:TL	WP:WD	LP:WP
Cephalonts:							
43	14	29	17	32	1:3.00	1:1.92	1:1.15
80	16	64	21	44	1:5.00	1:2.09	1:1.31
110	19	91	21	53	1:5.75	1:2.53	1:1.08
120	20	100	20	52	1:6.00	1:2.60	1:1.00
131	19	112	17	73	1:6.83	1:4.39	1:0.86
Sporonts:							
380	20	360	32	60	1:19.00	1:1.87	1:1.60

Table 7

Measurements of cephalonts and sporonts of *Stenophora juli* (Frantzius)

TL (μm)	LP (μm)	LD (μm)	WP (μm)	WD (μm)	LP:TL	WP:WD	LP:WP
Cephalonts:							
74	15	59	18	20	1:4.92	1:1.11	1:1.20
168	23	145	20	35	1:7.30	1:1.72	1:0.89
178	28	150	28	41	1:6.35	1:1.46	1:1.00
204	43	161	31	39	1:4.74	1:1.27	1:0.71
Sporonts:							
276	25	250	36	35	1:10.82	1:0.98	1:1.40
572	52	520	60	112	1:11.00	1:1.86	1:1.15

attached to the midgut epithelium (Fig. 12). The button ghaped epimerite, from 5 to 13 μm long, is persisting in cephalonts at various stages of maturation (Figs. 13, 14). The maximum length of mature cephalonts was up to 195 μm (Table 5). The endocyte is translucent and an ovoidal nucleus, with one karyosome, is well seen.

S p o r o n t s: Protomerite conical, frequently with recognizable flattened section to which the epimerite was attached (Fig. 15). Deutomerite spindle-shaped with well marked narrowed posterior region. Endocyte dark and not transparent due to dense paraglycogen granules.

Taxonomy

By the general form of the body this *Stenophora* somehow resembles *Stenophora typhloiuli* Golemansky and Taschev (1973) from the endemic troglobiont diplopod *Typhloiulus bureschi*, inhabiting some caves in Bulgaria (Golemansky and Taschev 1973). However both species differ by the length of sporonts, the shape and the length of protomerite. The sporonts of the discovered gregarine are over 500 μm long while sporonts of *S. typhloiuli* are only 350 μm long. The width of the deutomerite of *S. typhloiuli* is about 40 μm and the ratio WP:WD varies from 1.5 to 2.0. In case of the discovered gregarine the deutomerite of the sporont has the width of 128 μm and the ratio WP:WD varies from 1:2.51 to 1:3.36 (Table 5).

Due to these differences we consider that the eugregarine from *S. speleophilus* is a new species and we propose for it the name *Stenophora serboiuli* sp.n.

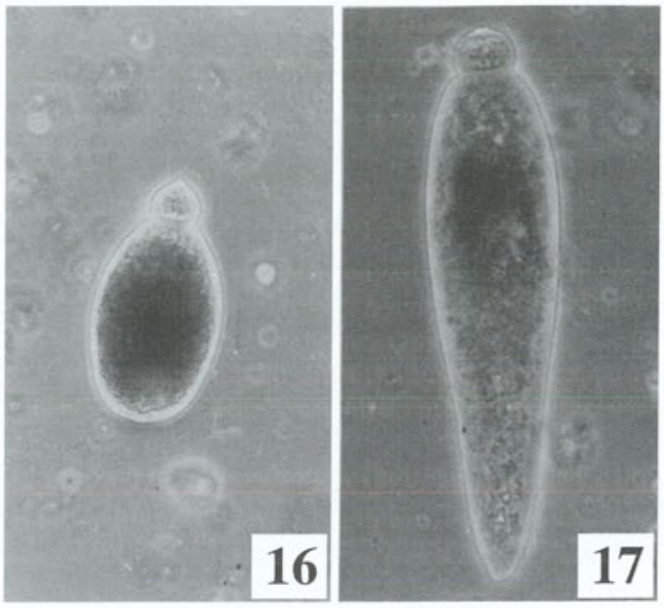
Stenophora bulgarosomae Golemansky
(Figs. 16-17)

Host: *Bulgarosoma meridionale* Tabacaru *(Diplopoda: Julidae)*.
Locality: Cave "Jagodinska Pestera" in Rhodopa Mountains; 27.IV.1980 (leg. V. Golemansky).

Morphology

C e p h a l o n t s: The young cephalonts are about 40 μm long while mature cephalonts have the length 80-131 μm (Table 6). Protomerite conical with slightly tapered top (Fig. 16). Deutomerite elipsoidal with dark endocyte. The nucleus is spherical, about 15-18 μm in diameter.

S p o r o n t s: Protomerite conical. Deutomerite elongated, narrowing toward the posterior end (Fig. 17). Nucleus spherical, with the diameter 18-20 μm, contains one karyosome. The largest observed sporont was up to 380 μm long.



Figs. 16-17 *Stenophora bulgarosomae* Golemansky: 16 - young cephalont (× 500), 17 - sporont (× 300)

Taxonomy

This is the first record of eugregarine infection from *Bulgarosoma meridionale*. This species is identified as *Stenophora bulgarosomae* Golemansky described from another troglobiont diplopod *Bulgarosoma bureshi* Verhoeff (Golemansky 1973). Both hosts are endemic cave diplopods in Bulgaria and, as they are phylogenetically very close and inhabit the same biotopes, we assume they are infected by the same gregarine *Stenophora bulgarosomae*.

Stenophora juli (Frantzius) Labbe

Host: *Pachyiulus hungaricus* (Karsch) *(Diplopoda: Julidae)*
Locality: Bistrica (near Sofia); 30.VI.1974; 10.V.1980 (leg. V. Golemansky).

Morphology

C e p h a l o n t s: The youngest cephalonts measured 74 μm while the mature ones up to 204 μm (Table 7). Epimerite of button type typical for the genus *Stenophora* and 2.5-3.0 μm in diameter. Protomerite conical. Deutomerite elongated, in some cases vermiform.

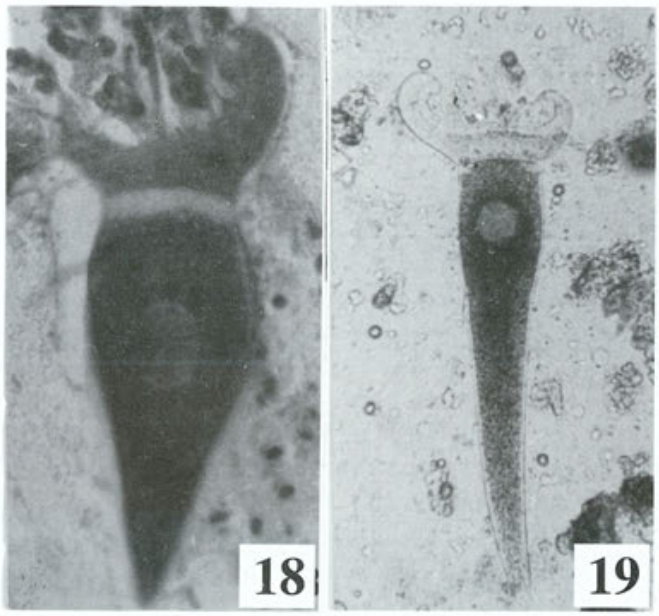
S p o r o n t s: Very long, cylindrical, with well visible pore on the top of the conical protomerite. The largest observed sporont was 572 μm long (Table 14).

Taxonomy

This is the first record of an eugregarine infection in a diplopod *Pachyiulus hungaricus*. The characteristics of this eugregarine indicate that it is a member of *Stenophora genus*. We identify this species as *Stenophora juli* (Frantzius) Labbe which has a wide range of hosts and is very polymorphic. We agree with Geus (1969) that *S. juli* is evidently a collective species and urgently needs more detailed investigations and possible revision.

***Grebneckiella gracilis* (Grebnecki) Bhatia**
(Figs. 18-19)

Host: *Scolopendra cingulata* L. *(Chilopoda, Scolopendridae)*.
Locality: Petric: 15.V.1977 (leg. V. Golemansky)



Figs. 18-19 *Grebneckiella gracilis* (Grebnecki): 18 - cephalont stained with Ehrlich's hematoxylin (× 500), 19 - sporont (× 200)

Morphology

Type of protomerite in cephalonts (Fig. 18) and in sporonts (Fig. 19) indicates that it is *Grebneckiella gracilis* known from *Scolopendra* spp. from Africa and France. The length of observed cephalonts was 600 μm (Fig. 18), while of sporonts 2800 μm (Fiq.19). This eugregarine is for the first time recorded in Bulgaria.

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ACTA PROTOZOOLOGICA

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Coccidian Parasites *(Coccidia: Eimeriidae)* of Domestic Rabbits *(Oryctolagus cuniculus domesticus* L.) in Syria

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Summary. Ten *Eimeria* species are reported from domestic rabbits (*Oryctolagus cuniculus domesticus L*) from Syria. Arranged by their prevalence they are: *E. perforans* (60%), *E. coecicola* (36%), *E. magna* (33.3%), *E. exigua* (25.3%), *E. irresidua* (24%), *E. matsubayashii* and *E. intestinalis* (12%), *E. piriformis* (10.6%), *E. media* (6.6%) and *E. stiedai* (4%). In 58 of 75 examined animals Coccidia were detected (total prevalence 77.3%). The authors supported the fact that an oocyst residuum is often present in the laboratory sporulated oocysts of *E. stiedai.*

Key words. Coccidia, *Eimeria*, domestic rabbit, Syria, oocyst morphometry, prevalence.

INTRODUCTION

The Coccidia are one of the most pathogenic parasites of domestic rabbit and especially of the young animals breed for meat production. Just now more than 11 Coccidian species are described from the domestic rabbit, six of which are highly pathogenic and cause severe coccidiosis and mortality (Pellerdy 1953, 1974, Pellerdy and Babos 1953, Ostler 1961, Mack 1962, Levine and Ivens 1970, Catchpole and Norton 1979, Norton et al. 1979).

The present study is the first attempt to learn the Coccidia and their prevalence in the domestic rabbit in Syria, where this animal is often raised in the private farms. To our knowledge only one article is published on domestic rabbit from the neigbouring countries, namely this of Kasim and Al-Shawa 1987 (Saudi Arabia).

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We hope that the present investigation will be a useful contribution to the knowledge on Coccidian fauna in Arabic countries and to their comparison with this of the other geographic regions.

MATERIAL AND METHODS

The samples of faeces from 75 domestic rabbits were collected during 1989-1991 from four regions of Syria (Table 1). The fresh faeces samples were kept in 2.5% potassium dichromate solution ($K_2Cr_2O_7$). Microscopical examination was done after flotation in NaCl solution by Fulleborn's method. For studuing the sporulation time of the oocysts the positive samples were incubated in thermostat at 24-25°C. Before that the faeces were macerated in fresh water and placed in Petri dishes covered by humid filter paper. The observations and the photographs were made by a microscope NU-2 (Zeiss, Jena) with magnifications from 100 to 400 x.

The variation of the oocyst size is calculated by the equation

$$x = \frac{\Sigma x}{n}, \quad C.V. = \frac{S \cdot V \cdot 100}{\bar{x}} \text{ and } S.D. = \sqrt{\frac{\sum (x = \bar{x})^2}{n}},$$

where: n - is the number of oocysts measured, x - the size of the oocyst,

Table 1

Localities and number of examined and invased samples

Locality	Time	Total number	Positive samples
Daraa	IX , 1989	5	4
	VI , 1990	10	5
	VIII , 1990	11	11
	X , 1990	3	3
	I , 1991	4	1
Damascus	IX , 1989	2	1
	VI , 1990	2	2
	VIII , 1990	7	7
	X , 1990	1	1
	I , 1991	8	6
Homs	IX , 1989	4	4
	VI , 1990	1	0
	VIII , 1990	2	2
	I , 1991	2	1
Latakia	VI , 1990	4	4
	VIII , 1990	4	4
	I , 1991	5	2
Total		75	58

$\bar{x}$ - arithmetical mean size, S.D. - standard deviation, C.V. - coeficient of variations, m - standard error.

The other abbreviations used in the text and in the tables are: L - length of the oocyst, W - width of the oocyst, M - mean and Lim. - limits of the variation.

RESULTS

In the examined domestic rabbits (Oryctolagus cuniculus domesticus L.) from Syria the next ten species of Coccidia were found:

Eimeria perforans (Leuckart, 1879) Sluiter a. Swellengrebel, 1912 (Figs. 2, 3)

Oocyst morphometry: The oocysts are often ellipsoid, sometimes ovoid, colourless, with a smooth colourless wall, composed of two layers (0.9-1.25 μm) thick. The micropyle is absent but rarely can be seen a fin structure similiar to micropyle. Oocyst residuum is present, without a polar granulein the oocysts. The measurments of the oocysts and their variations are present at Table 2.

The sporocysts are ellipsoidal, 8.75-11.5 by 4.5-6 μm (mean 9.6-5.2 μm). L/W ratio vary from 1.58 to 2.2

Table 2

Eimeria perforans: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 22.12 + 0.21	1.15 – 2.16	3.28	14.82	15.5–32.5	251
W: 14.84 + 0.11	M = 1.48	1.68	11.32	11.75–22.5	251

Table 3

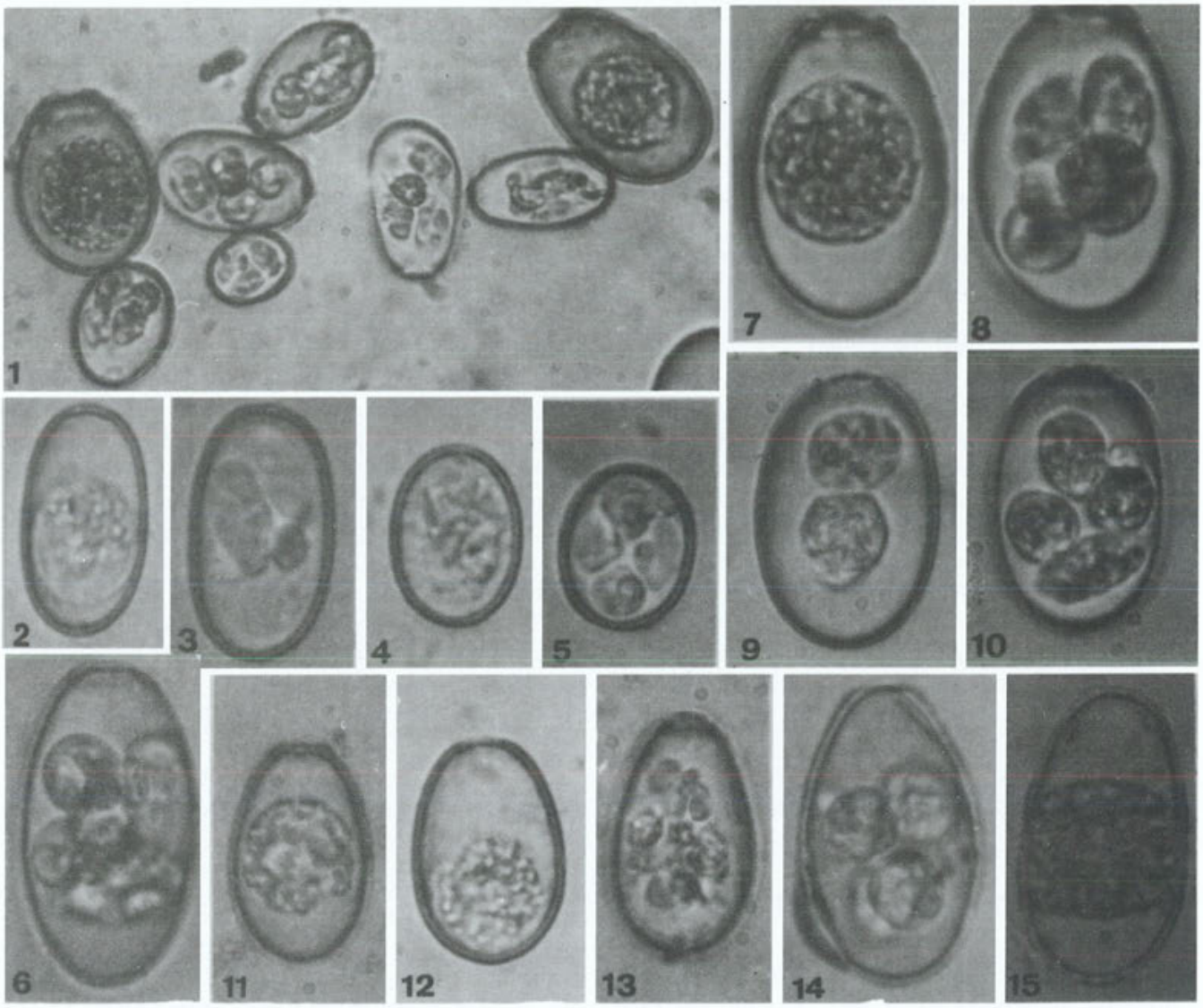
Eimeria exigua: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 17.99 + 0.17	1–1.5	1.91	10.61	14–23	119
W: 14.29 + 0.12	M = 1.25	1.32	9.23	11.25–18	119

Table 4

Eimeria coecicola: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 32.13 + 0.18	1.3–2.14	2.06	6.41	26.25–38.75	128
W: 18.45 + 0.13	M = 1.74	1.48	8.02	14–25	128



Figs. 1–15. *Eimeria* species from domestic rabbit from Syria. 1 – Mixed intensive invasions of a domestic rabbit with four different Eimerian species (x 850), 2–3 – *E. perforans* (Leuckart) Sluiter and Swellengrebel (x 1100): 2 – unsporulated oocyst, 3 – sporulated oocyst, 4–5 – *E. exigua* Yakimoff (x1400): 4 – unsporulated oocyst, 5 – sporulated oocyst, 6 – *E. coecicola* Kheissin – sporulated oocyst (x 1500), 7–8 – *E. magna* Perard (x 1400): 7 – unsporulated oocyst, 8 – sporulated oocyst, 9–10 – *E. irresidua* Kessel and Jankiewicz (x 1000): 9 – phase of two sporoblasts, 10 – sporulated oocyst, 11 – *E. matsubayashii* Tsunoda – unsporulated oocysts (x 1100), 12 – *E. media* Kessel – unsporulated oocyst (x 1200), 13 – *E. piriformis* Kotlan and Pospesch – sporulated oocyst, 14 – *E. intestinalis* Kheissin (x 1400), 15 – *E. stiedai* (Lindemann) Kisskalt and Hartmann – unsporulated oocyst (x 1400)

(M = 1.86). Sporocyst residuum and Stieda body are present. Sporozoites are elongate.

Prevalence: *E. perforans* was found in 45 animals (60%).

Eimeria exigua Yakimoff, 1934 (Figs 4, 5)

Oocyst morphometry: The oocysts are spherical or subspherical, colourless or lightpink, with smooth wall devoid of a micropyle. Oocyst residuum and polar granule in sporolated oocysts are absent. The dimensions of the oocysts and their variations are present at Table 3.

The sporocysts are ovoid, 6.25-10 by 4.25-6 μm .

Prevalence: *E. exigua* was found in 19 animals (25.3%).

Eimeria coecicola Kheissin, 1947 (Fig. 6)

Oocyst morphometry: The oocysts are cylindrical or ellipsoid, rarely ovoid, light brown

colour, with a microple at narrow end (3.75-7.5 μm). Oocyst wall is smooth and thicker around a microple. Oocyst residuum is present but polar granule is absent. Our measurments of the oocysts and their variations are present at Table 4.

The sporocysts are ellipsoid to ovoid, with granular sporocyst residuum and Stieda body. Their dimensions vary from 12-13 by 5.75-7.5 μm.

Prevalence: *E. coecicola* was found in 27 animals (36%).

***Eimeria magna* Perard, 1925** (Figs. 7, 8)

Oocyst morphometry: The oocysts are ovoid, sometimes ellipsoid, frequently brownish or yellowish orange, with a well formed micropyle at the end (5-8 μm). Around the micropyle a collar - like protrusion is well visible. The oocysts are with large residuum (8.75-10.5 μm), polar granule is absent. The measurments of oocyst and their variations are present at Table 5.

The sporocysts are ovoid, without Stieda body. Dimensions of the sporocysts vary from 13-16.25 by 6.25-8.7 μm (M 14.4-7.75 μm).

The sporozoites are elongate, lie length - wise head to tail.

Prevalence: *E. magna* was found in 25 animals (33.3%).

***Eimeria irresidua* Kessel et Jankiewiez, 1931** (Figs. 9, 10)

Oocyst morphometry: The oocysts are ellipsoidal, sometimes ovoid, with curvature at each pole. At one end there is prominent micropyle (3.75-8.75 μm, with a mean value 6 μm). Oocyst wall is smooth, yellow and composed of two layers (1.25-1.5 μm) in thickness. Oocyst residuum and polar granule are absent. The measurments of the oocysts and their variations are present at Table 6 .

The sporocysts are elongate ovoid with a small Stieda body and large residuum. The sporocyst measurments are 12-17.5 by 7.5-10 μm, with a mean 13.75 by 8.25 μm. The sporozoites arranged head to tail, with two large refractile globules.

Prevalence: *E. irresidua* was found in 18 animals (24%).

***Eimeria matsubayashii* Tsunoda, 1952** (Fig. 11)

Oocyst morphometry: The oocysts are broadly ovoid with yellowish brown colour. The wall is smooth and has a micropyle. Around the micropyle it is little thicker. The diameter of the micropyle is 5.95 μm. Oocyst residuum is present but no polar granule. The dimensions and their variations are given at Table 7.

The sporocysts are ovoid (14-15 by 7-7.5 μm) with a Stieda body and granular residuum, the sporozoites have a clear globule at the large end.

Prevalence: *E. matsubayashii* was found in 9 animals (12%).

***Eimeria media* Kessel, 1929** (Fig. 12)

Oocyst morphometry: The oocyst shape is ovoid to ellipsoid. The oocyst wall is light yellowish, smooth and composed of two layers (0.9-1.1 μm), with micropyle (5.75-7 μm). Oocyst residuum is present, 5.75-6.25 μm in diameter. Polar granule is absent. The measurements and their variations are present at Table 8.

The sporocysts are elongate ovoid (13-14 by 7-8.25 μm), with a small residuum and one clear globule at the large end of the sporozoites.

Prevalence: *E. media* was found in 5 animals (6.7%).

***Eimeria piriformis* Kotlan et Pospesch, 1934** (Fig. 13)

Oocyst morphometry: The oocysts are pear-shaped, yellowish brown. Oocyst wall is smooth and composed of two layers with a micropyle on the narrow end. Oocyst residuum and polar granule are absent. The measurements and their variations are present at Table 9.

The sporocysts are ovoid (11.25-12 by 8 μm), with granular residuum and Stieda body. The sporozoites are with a clear globule at the large end.

Prevalence: *E. piriformis* was found in 8 animals (10.6%).

***Eimeria intestinalis* Kheisin, 1948** (Fig. 14)

Oocyst morphometry: The oocysts are pear-shaped, greenish-brown, with distinct micropyle at the narrow end. Oocyst wall is composed of two layers. Oocyst residuum present, 4-6 μm in diameter. The polar granule is absent. The measurements and their variations are present at Table 10.

The sporocysts are ovoid to elongate, 9.5-15 by 6.25-9 μm (mean 13 by 7.5 μm), with small residuum and Stieda body.

Table 5

Eimeria magna: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 34.05 + 0.18	1.25–1.82	2.24	6.57	28.75–41.25	155
W: 2.64 + 0.15	M = 1.5	1.85	8.17	18.65–27	155

Table 6

Eimeria irresidua: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 35.33 + 0.29	1.23–1.76	3.19	9.02	30–43.75	128
W: 24.03 + 0.18	M = 1.47	1.93	8.03	20–29	128

Table 7

Eimeria mastubayashii: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 30.41 + 0.5	1.2–1.9	3.43	11.27	21.25–35	48
W: 21.09 + 0.35	M = 1.44	2.53	11.99	15.5–26.25	48

Table 8

Eimeria media: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 27.71 + 0.79	1.33–1.92	4.16	15.01	18.75–35	28
W: 18.22 + 0.57	M = 1.52	3.04	16.68	13.75–23.75	28

Table 9

Eimeria piriformis: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 30.32 + 0.4	1.25–1.68	2.67	8.8	25–35	37
W: 20.11 + 0.19	M = 1.5	1.13	5.61	17–22.5	37

Table 10

Eimeria intestinalis: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 31.23 + 0.63	1.25–1.85	3.19	10.2	23.5–36.25	26
W: 19.13 + 0.26	M = 1.63	1.31	6.84	17.5–21.25	26

Table 11

Eimeria stiedai: Oocyst dimensions and their variations

$\bar{x} \pm m$	L/W	S.D.	C.V.	Lim	n
L: 31.52 + 0.54	1.36–1.81	2.91	9.23	22.5–36.5	29
W: 20 + 0.36	M = 1.57	1.98	9.8	15–23.75	29

P r e v a l e n c e: *E. intestinalis* was found in 9 animals (12%).

Eimeria stiedai (Lindemann, 1865) Kisskalt et Hartmann, 1907 (Fig. 15)

O o c y s t m o r p h o m e t r y: The oocysts are elongate ovoid, sometimes ovoid asymmetrical, colorless or yellowish. The wall is smooth and composed of two clear layers (1.25-1.5 μm). The micropyle is indistinguishable but when it is visible it is about 4-5 μm wide. Oocyst residuum more frequently present (about 4 μm in diameter), but sometimes is absent. The polar granule is absent. The measurements and their variations are present at Table 11.

The sporocysts are elongate ovoid, 12.5-15.5 by 6.25-10 μm (mean 13.6 by 7 μm), with a fine residuum and Stieda body.

P r e v a l e n c e: *E. stiedai* was found in 3 animals (4%).

Table 12

Number of Eimerian species in the invased domestic rabbit from Syria

No. of *Eimeria* species	Number of invased rabbits	Prevalence %
0	17	–
1	10	13.3
2	13	17.3
3	19	25.3
4	9	12.0
5	5	6.7
6	1	1.3
7	1	1.3
8	–	–
9	–	–
10	–	–
Total	75	73.3

CONCLUSION

In general 10 species of *Eimeria* were established in the examined domestic rabbits (*Oryctolagus cuniculus domesticus* L.) from Syria. Arranged by their prevalence they are: *E. perforans* (60%), *E. coecicola* (36%), *E. magna* (33.3%), *E. exigua* (25.3%), *E. irresidua* (24%), *E. matsubayashii* and *E. intestinalis* (12%), *E. piriformis* (10.6%), *E. media* (6.6%) and *E. stiedai* (4%). Six of them are known as pathogenic for domestic rabbits, namely: *E. irresidua, E. magna, E. coecicola, E. media, E. piriformis* and *E. stiedai* (Smetana 1933, Kheissin 1948, Lund 1949, Pellérdy and Babos 1953, Pellérdy and Duzz 1970, Levine and Ivens 1972, Norton et al. 1979).

Of all the 75 examined animals coccidian oocysts were detected in 58, hence the total prevalence is quite high (77.3%). Mixed invasion with 2 or 3 coccidian species was most frequent (Table 12).

E. stiedai was a most rare species in our material. We found it in the fecal samples only of 3 examined animals (4%). We think that this very low prevalence is not real because we didn't explore the liver material of the

examined domestic rabbits. In cases of lower invasion of the rabbits with *E. stiedai* it is quite occasional to obbserve its oocysts in the fecal samples.

Our investigation on the sporulation of *E. stiedai* proved the dates of Norton et al.(1977) about the existence of an oocyst residuum in the big part of the laboratory sporulated oocysts of this species.

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ACTA
PROTOZOOLOGICA

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A Haplosporidian *Haplosporidium meligethi* sp.n., and a Microsporidian *Nosema meligethi* I. et R., Two Protozoan Parasites from *Meligethes aeneus* F. *(Coleoptera: Nitidulidae)*

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Summary. A new haplosporidian *Haplosporidium meligethi* sp. n. is described from the rape blossom beetle (*M. aeneus* F.) collected in Minsk (USSR), Hartola (Finland) and Poznań (Poland). The life cycle of *H. meligethi* sp. n. goes through the stage of plasmodium with plasmotomy division ending with the production of cysts (pansporoblasts) containing variable number of spores. Spores measure 3.2-4.8 by 1.6-2.6 μm and do not contain polar filaments. These features separate this haplosporidian clearly from a microsporidian *Nosema meligethi* I. et R., known from the same host insect. Data on development stages of *N. meligethi* and the tissues attacked in the host insect are presented. Both parasites were observed only in adult insects and the reasons of this require special studies.

Key words. *Haplosporidium meligethi* sp.n., *Nosema meligethi*, parasites, coleopterans, *Meligethes aeneus.*

INTRODUCTION

While continuing our studies on microsporidiosis of the rape blossom beetle (*Meligethes aeneus* F.) caused by *Nosema meligethi* I. et R. (Hokkanen and Lipa 1991 a, b; Lipa and Hokkanen 1991) we discovered a new haplosporidian species parasitic in beetles sampled at Minsk (USSR), Hartola (Finland) and Poznań (Poland). This haplosporidian has spores and life cycle quite different from *N. meligethi* and we report about our findings in this paper.

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MATERIAL AND METHODS

Adults of *M. aeneus* for this study were collected during August-September of 1989, June-September of 1990 and May of 1991. The beetles were placed individually into a small drop of water on a microscopic slide and dissected with needles, or their bodies were crushed (grounded) with a rounded glass stick in order to release the spores from infected tissues. The slides were then examined with a compound microscope at the magnification from 160x to 640x. The protozoan spores were easily recognized due to their uniform size and refractile wall, as compared to frequently present yeast cells having variable shape and size and being in the phase of budding.

The life cycles of the recorded protozoans were studied on smeared preparations of *M. aeneus* tissues which were air dried, fixed in methanol for 2 min and stained with 0.1% Giemsa's stain for 3-12 h. The holotype slide of *Haplosporidium meligethi* sp. n. is deposited in the collection of the Institute of Plant Protection in Poznań; the paratype slides are deposited in the Department of Agricultural and Forestry Zoology, University of Helsinki.

RESULTS

Protozoan infections in *Meligethes aeneus* populations

During 1989-1991 about 13939 adults of *Meligethes aeneus* collected in 12 European countries were microscopically examined and a microsporidian infection caused by *Nosema meligethi* was found in 561 adults (Lipa and Hokkanen 1991). However, in three populations collected in Minsk (USSR), Hartola (Finland) and Poznań (Poland) another protozoan infection was discovered caused by a protozoon producing polysprous cysts and therefore differring from *N. meligethi.* At first we believed that this species was a microsporidian belonging to a *Pleistophora*-complex (Canning et al. 1991) but at more detailed studies it was found that it is a haplosporidian which we describe here as a *Haplosporidium meligethi* sp.n.

The level of infection caused by *H. meligethi* sp.n. was low as compared with *N. meligethi*, and the haplosporidian infection was recorded only in 3 samples out of over 312 checked (Table 1).

Table 1

Prevalence of infections caused by *Nosema meligethi* and *Haplosporidium meligethi* sp. n. in three populations of *Meligethes aeneus*

Locality and date	No. of adults examined	% of infection by *N. meligethi*	% of infection by *H. meligethi*
Mińsk (USSR), 15.8.89	46	17.4	4.3
Hartola (Finland), 16.6.90	94	13.8	3.1
Poznań (Poland), 29.5.91	28	0	3.5

Morphology and Develoment of *Haplosporidium meligethi* sp.n.

The spores of a newly recorded haplosporidian occur in cysts (pansporoblasts) in variable number from 8 to 32 (Figs. 1, 7, 10). The spores in water mount (Fig. 2) or stained with Giemsa (Figs. 8, 9, 10), have a peculiar appearance, quite different from spores of the microsporidian *Nosema meligethi.* They have a "bivalve" structure, with apparently two nuclei located on both sides of the spore, while in the central part of spore, a vacuole is located. Therefore, the spores in water, as well as on stained preparations, have the central part lighter than the outer part. In spite of various efforts it was not possible to cause the extrusion of the polar filaments and it is assumed that the spores are lacking of filaments what is characteristic for Haplosporidia.

The life cycle of the new haplosporidian goes through a multinucleate plasmodium stage (Figs. 3, 4), which by plasmotomy produces binucleate merozoites; these turn into sporoblasts (Figs. 5, 6), and then into spores grouped in pansporoblasts (Figs. 1, 7, 10).

The fixed and stained spores were 3.20-4.80 μm long and 1.60-2.48 μm wide (Table 2).

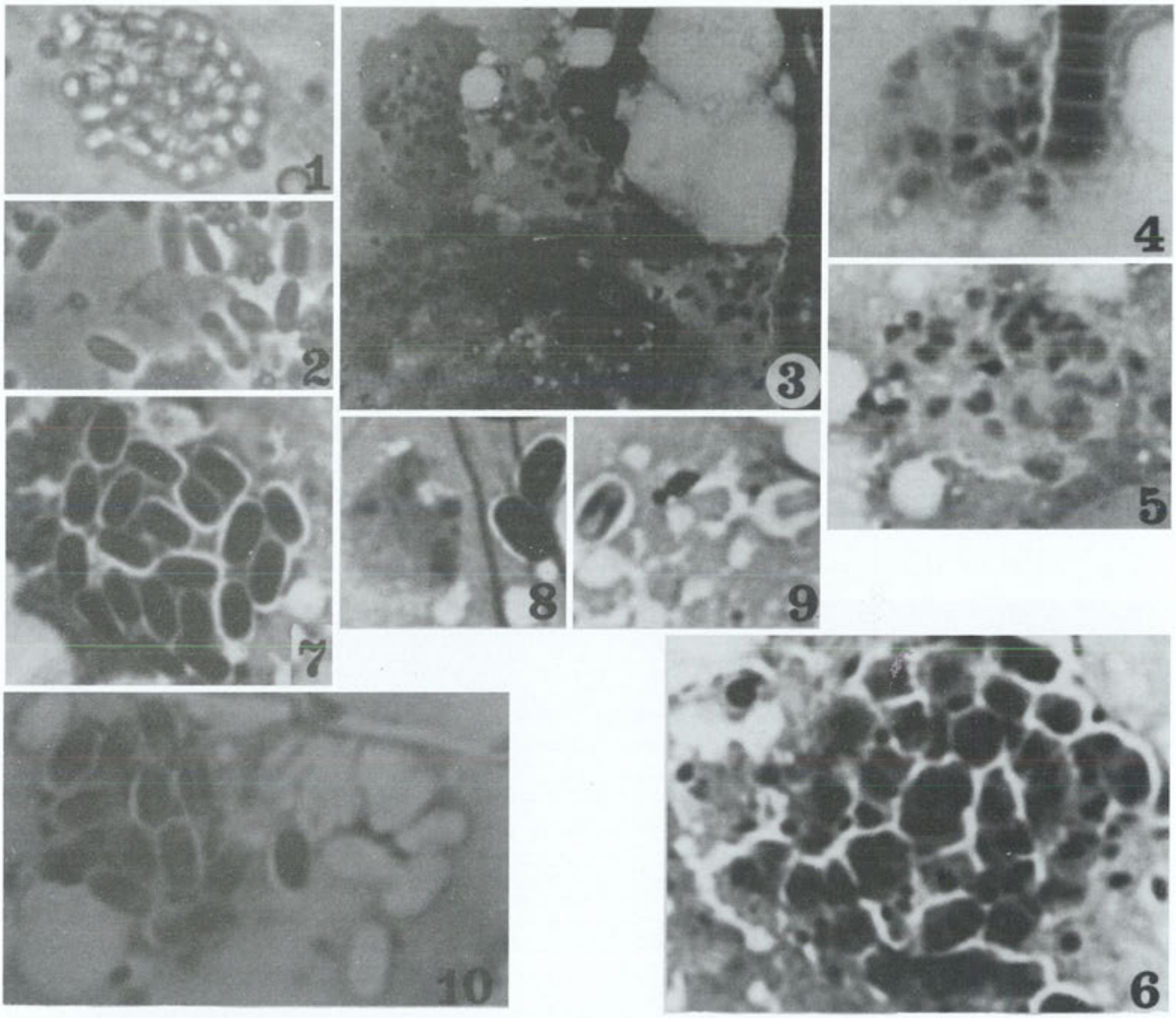
The infection seems to be limited to the intestine of *M. aeneus*, but the full pathological history is not available due to the scarce material - 6 infected adult beetles out of over 13938 microscopically examined individuals. This was also the reason for not being able to perform electron microscope studies, which would allow to make a complete generic identification of this haplosporidian.

So far this is the only haplosporidian known in beetles (Coleoptera) since Weiser (1970) reassigned *Haplosporidium typographi* (Weiser 1954) to the microsporidia as *Chytridiopsis typographi* parasitic in beetles *Ips typographi* L. and *Dendroctonus pseudotsugae* Hopkins. Due to the characteristic life cycle and spore morphology of the protozoan recorded in *Meligethes aeneus* we consider that the haplosporidian

Table 2

Comparison of dimensions (in μm) of 50 stained spores of *Haplosporidium meligethi* sp. n. and *Nosema meligethi*

Species	Range Length Width	Mean (n = 50)	SD
H. meligethi sp. n.	3.28 – 4.80 x 1.60 – 2.64	3.78 x 2.10	0.4090/0.3410
N. meligethi I. et R.	3.12 – 4.48 x 1.76 – 2.40	3.73 x 2.16	0.4388/0.2224



Figs. 1-10. *Haplosporidium meligethi* sp. n.: 1 - Spores inside pansporoblast (unstained in water); 2 - Free spores with visible peculiar internal structure (in water, phase contrast); 3-4 - Plasmodial stages (Giemsa stain); 5-6 - Plasmodia in the process of plasmotomy (Giemsa stain); 7 - Pansporoblast with 20 spores (Giemsa stain); 8 - Quadrinucleate plasmodium and nature spores (Giemsa stain); 9 - Stained spore with clearly visible two nuclei in opposite position; 10 - Spores with characteristic less intensively stained central part; on the right side a group of empty spores is seen (Giemsa stain)

found by us is a new species and therefore we propose the name *Haplosporidium meligethi* sp.n. for it.

Development and Pathogenicity of *Nosema meligethi* I. et R.

Our observations confirm the data given by Issi and Raditscheva (1979) in the original description of *N. meligethi* and presented in the form of drawings. In this paper we present several photographs of developmental stages and tissues attacked by the pathogen.

Spores of *N. meligethi* occur singly (Figs. 11, 12) and look quite different from spores of *Haplosporidium meligethi* sp.n. Meronts with diplokaryotic (Fig. 13) as well as with single nuclei (Fig. 14) were observed. Quadrinucleate meronts (Figs. 15, 16) frequently have the nuclei arranged in a diplokaryotic way. Multinucleate plasmodia were not observed among developmental stages of *N. meligethi.*

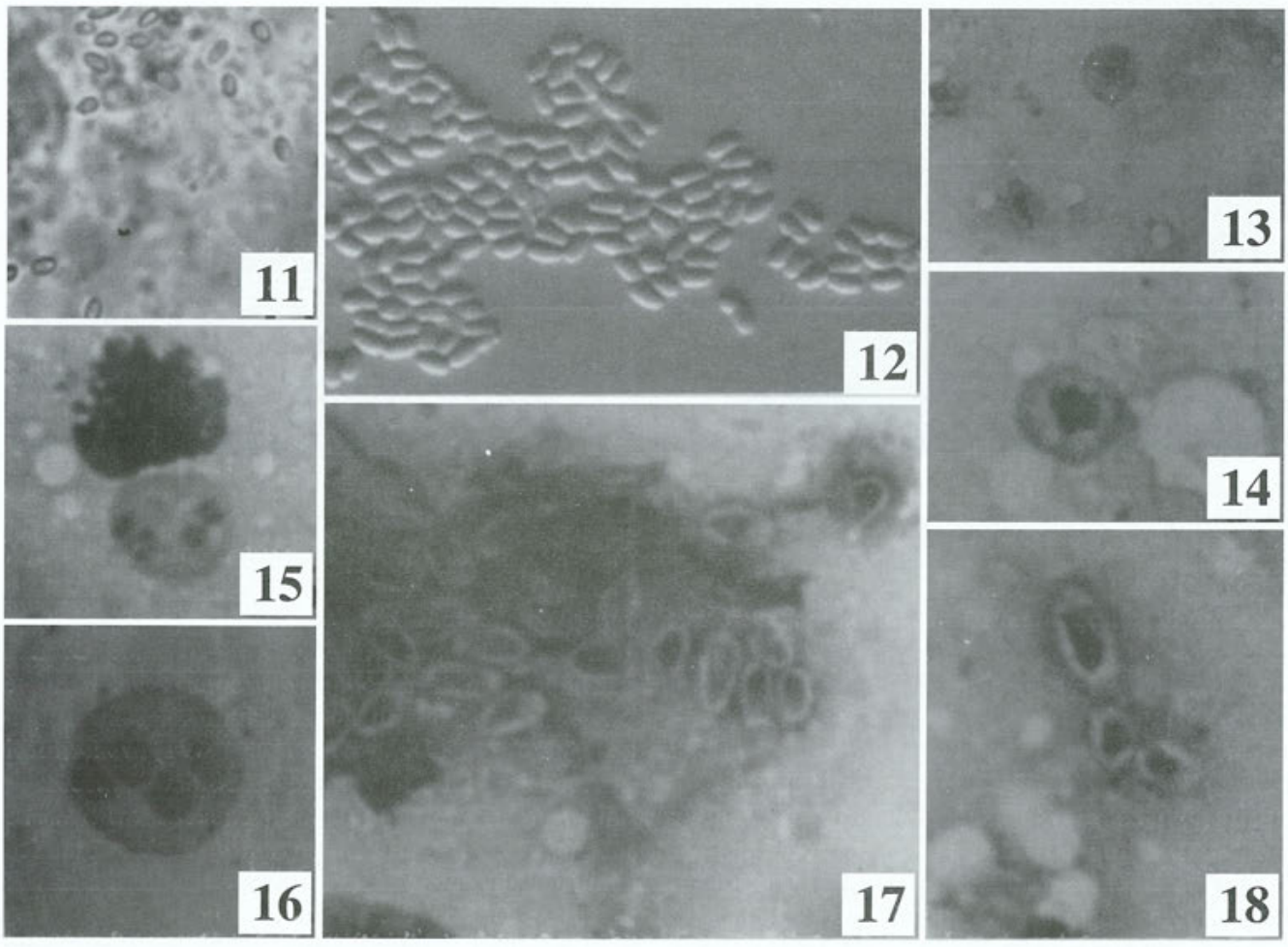
Fixed and stained spores of *N. meligethi* were 3.12-4.48 μm long and 1.76-2.40 μm wide (Table 2). Issi and Raditscheva (1979) for living spores gave the

dimensions 4.0-4.8 x 2.0-3.0 μm and for fixed and stained spores 3.6-4.8 x 2.4 μm. As seen on Figs. 17 and 18, there is a significant variation in spore dimensions which was also indicated on drawings by Issi and Raditscheva (1979).

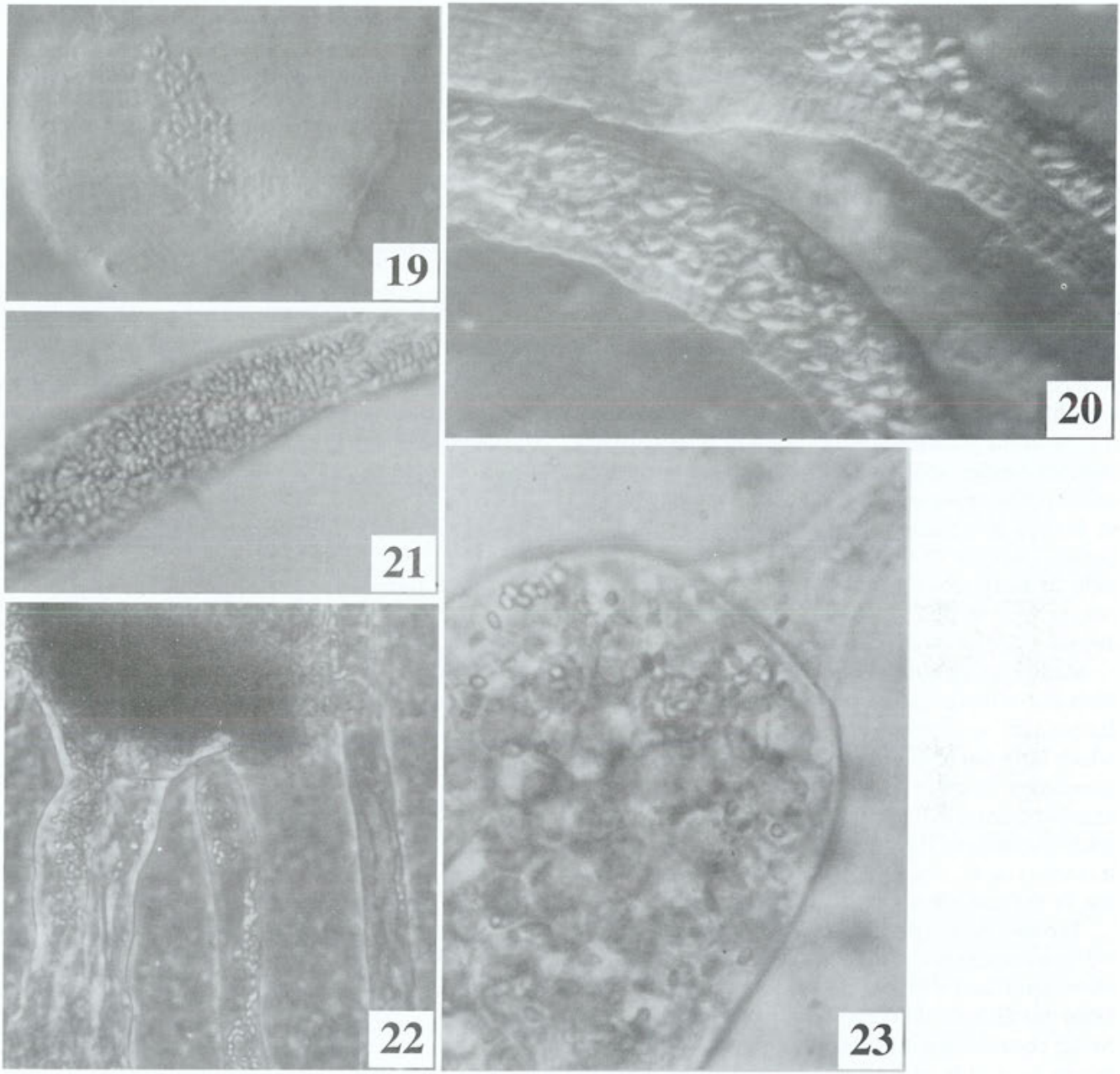
Infection was observed only in adults and larvae checked from several populations were free from the parasite's attack. While reporting on tissues infected, Issi and Raditscheva (1979) mentioned only fat body attacked. In our microscopic observations we recorded the presence of spores in muscles (Figs. 19, 20), Malpighian tubules (Fig. 21), nerve commissures and ganglions (Fig. 22) and in female gonads (Fig. 23). It is seen that intensity of tissue attack was frequently very high and hyperthropy and other pathological changes were observed. This explains the reasons of high mortality among overwintering infected adults. The infection of gonads by *N. meligethi* confronted with the lack of infection among larvae is quite puzzling and requires special studies.

DISCUSSION

The rape blossom beetle (*M. aeneus*) was known as host for *Nosema meligethi* I et R. recorded at first in the neighborhood of Leningrad (Issi and Raditscheva 1979), and later found in Pieksamaki (Finland) (Hokkanen et al. 1988). In our extensive survey conducted during 1989-1990 in Finland (Hokkanen and Lipa 1991 a, b) we have confirmed the presence of this pathogen in *M. aeneus* populations in southern part of Finland, and discussed the reasons of its more frequent occurrence in the non rape growing areas. In further studies we have also shown the occurrence of *N. meligethi* in populations of its host in Austria, Czechoslovakia, Denmark, Finland, Hungary, Poland and the Soviet



Figs. 11-18. *Nosema meligethi* I. & R.: 11 - Spores in water mount (unstained); 12 - Spores in water (Nomarski's contrast); 13 - Meront with diplokaryotic nucleus (Giemsa stain); 14 - Uninucleate meront (Giemsa stain); 15-16 - Quadrinucleate meronts (Giemsa stain); 17 - A group of stained spores; 18 - One large and two small stained spores



Figs. 19-23. *Nosema meligethi* I. & R.: 19-20 - Spores in muscles; 21 - Spores in Malpighian tubules; 22 - Spores in nerve commissures and ganglions; 23 - Spores in female gonad

Union. On the other hand, the pathogen was absent in sampled populations from Germany, Norway, Sweden, Switzerland and United Kingdom (Lipa and Hokkanen 1991).

Altogether, during 1989-1991 312 samples with 13938 beetles were microscopically examined and the mean infection level caused by *N. meligethi* was 4.02%, but is some individual populations the infection level was over 20%. The mean infection level caused by *Haplosporidium meligethi* sp.n. was 0.043% but in individual populations ranged from 3.1 to 4.3% (Table 1).

The fact that infection caused by *H. meligethi* sp.n. was recorded only in three populations and at such a low level indicates that this haplosporidian apparently plays a minor role as a mortality factor in populationbs of *M. aeneus* as compared with *N. meligethi.*

Both protozoans have spores of similar dimensions

but they greatly differ in their life cycles and the spore appearance. These features easily allow to distinguish both species basing on spore morphology and to asses their occurrence and prevalence in various host populations.

H. meligethi sp.n. evidently affects only the gut while *N. meligethi* causes a general infection as its spores were observed in fat body, muscles, Malpighian tubules, nerves and gonads. Of special interest is fact that infections caused by both protozoans were observed only in adult beetles. None of the over two thousand microscopically examined larvae was found to harbor one of the studied parasites. This is quite puzzling, as *N. meligethi* infection was observed in female gonads, and this allows to assume that vertical (congenital or transovarial) transmission of infection to progeny of infected beetles should occur. Also Issi and Raditscheva (1979) reported infection with *N. meligethi* only in beetles but these authors did not examined the larvae. The case of a microsporidian infection limited only to adult insects is known in the literature and referes to infection or honeybee (*Apis mellifera* L.) caused by *Nosema apis* Zander.

Studies on epizootiological aspects of microsporidian and haplosporidian infections in *M. aeneus* populations will be continued considering also the ways in which both microsporidians are spreading within one generation (horizontally) or from one generation to another (vertically). Such studies are needed for better understanding of the role both protozoans play in the dynamics of *M. aeneus* populations and their possible use in the integrated control of this pest.

The taxonomic position of *Haplosporidium meligethi* sp.n. require some future studies. Its placement into Haplosporidia (=Haplosporea) seems to be fully justified by distinct differences with the Microsporidia. While consulting with Prof. E. Canning the taxonomic position of this protozoon she stated that due to peculiar structure of spore and type of development it cannot be assigned to any of the presently known genera of the *Pleistophora*-complex characterized by producing polysporous pansporoblasts (Canning et al. 1991, Issi 1986, Larsson 1988). However, as pointed out by Brooks (1974, 1988) and Sprague (1979, 1982) taxonomy of Haplosporidia urgently need some basic reconsideration. As an example of that may serve fact that the protozoon originally described by Weiser (1954) from *Ips typographi* as *Haplosporidium typographi* has been later reassigned to the microsporidian genus *Chytridiopsis* as *C. typographi* (Weiser 1970). Brooks (1974, 1988) emphasizes that the taxonomic status of two more recently described haplosporidian species *H. tipulae* Huger (1961) and *H. simulii* Beaudoin et Wills (1968) may be questioned. While considering the possibility of creating a new genus for the described species we at the present time designate it as *Haplosporidium meligethi* sp.n.

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