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SPECIFIC FEATURES OF RESTING CYSTS MORPHOLOGY OF LIMNIC HETEROTRICHOUS SPECIES CILIATES *BLEPHARISMA LATERITIUM* AND *STENTOR ROESELII*

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ABSTRACT

The following light microscopy study is dedicated to the structural description of resting cysts from two limnic heterotrichous species of ciliates, *Blepharisma lateritium* (Ehrenberg, 1831) Stein, 1859 and *Stentor roeselii* Ehrenberg, 1835. Both limnic samples were taken from the same location in Slovakia. In addition to standard observations during the encystation process of *B. lateritium* (changes in shape, volume, size; resorption of ciliature and others), we identified a specific structure around the encysting cells known as the „paper layer". The most significant feature is the morphology of ectocyst and the presence of specific conical-shaped plug (escape apparatus). In addition to *B. lateritium* resting cysts, we described the resting cysts structure of *S. roeselii* for the first time. We recorded the unusual number of pillars visible in the form of thin hem just below the cyst surface. The outer layer (most likely ectocyst) appeared very striated, distally marked by a mucous layer. Unambiguously, this is the most conspicious feature of the cystic wall. The literary data suggest that there is still little information about these two species, as well as about cyst morphology in ciliates in general. This work can provide the basis for further research.

INTRODUCTION

The important part of the life cycle of the most ciliates is the formation of the resting cysts – encystation. The antagonistic process of encystation is excystation (Verni & Rosati 2011). These processes represent a strategy against unfavourable enviromental factors. The factors that control the encystation-excystation cycle (further than E-E cycle) in ciliates are very diverse (Gutiérrez et al. 2001; Verni & Rosati 2011). Many authors consider dessication and lack of a food source as the most universal exogenous inducers of the encystation process. Other factors such as day length, the presence of predators in the environment, massive overgrowth, temperature, salinity and pH, stimulate both processes, especially in limnic species (Mulish & Hausmann 1989; Calvo et al. 2003; Verni & Rosati 2011). A number of current studies with descriptions of resting cysts and E-E cycles in ciliates are available in the literature.

According to Foissner et al. (2007) and Verni & Rosati (2011), the detailed morphological and physiological information on resting cysts were available until 2007 for less than 40 species of ciliates. Despite the growing interest from this period, the issue of resting cysts still offers many opportunities

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for interesting exploration (Foissner et al. 2007; Foissner 2009; Verni & Rosati 2011).

The resting cysts morphology of two limnic species *Blepharisma lateritium* and *Stentor roeselii* was basically described in this study. We studied only live observations of resting cysts structure of both species.

Several authors have been devoted to the resting cysts morphology of the genus *Blepharisma*. The resting cysts of species *B. lateritium*, *B. stoltei* and *B. undulans* had already been basically described by Repak (1968), Walsh & Isquith (1979), Mulisch & Hausmann (1989) and Foissner et al. (1992). For example, the progress of the encystation process and resting cysts were described in Repak (1968) for *B. stoltei* and in Cavaleiro et al. (2017) for *B. sinuosum*.

The resting cysts of the species *S. roeselii* had not been previously described in any work. The resting cysts of the genus *Stentor* have been examined very poorly. This information is summarized in Tartar (1961) and Foissner et al. (1992). The information is only for the species *S. coeruleus*, *S. niger* and *S. polymorphus*. Even Foissner questioned the correct identification of the resting cysts of the species *S. polymorphus*, since the data are from the works of Stein (1867) and have not yet been verified by other current research. Data on the *S. roeselii* are still lacking. We have described the basic structure of resting cysts in light microscopy for the first time.

MATERIAL AND METHODS

Blepharisma lateritium and *Stentor roeselii* were isolated from the same limnic sample taken from Váh River in the locality of Lisková (near Ružomberok), Northern Slovakia (N 49° 5' 3'', E 19° 21' 9'') on 8. 4. 2016. The samples were collected from a littoral zone and transported in glass bowls (bottles) with a volume of 250 – 300 ml to laboratory conditions. Subsequently, these original samples were used for identification and to obtain a raw culture of the studied ciliates. The samples were investigated in a laboratory on the second and third day after field collecting. During this time, the highest abundance of specimens of both species was also recorded.

Later, a part of the original culture and tap water (in a ratio of 1 : 1) was transferred to Erlenmeyer flasks with a volume of 250 ml with the aim of inducing encystation.

The main factor that induced encystation was the loss of natural conditions associated with a gradual depletion of a food source in the culture dishes. In about 2 – 3 days (in the case of a species *B. lateritium*) and about 6 – 7 days (in the case of a species *S. roeselii*), the cells began to encyst.

Trophic cells, the encystation process and the resting cysts structure were observed using only the optical microscope Leica DM 1000 at low (100 – 400 ×) and high (1000 ×; with immersion) magnifications. In vivo measurements of cystic cells were conducted at magnifications of 100 – 1000 ×. All images were captured using a Leica EC3 camera. Schemes were graphically processed in the CorelDraw X6 and X7 program.

Identifications of the species of *B. lateritium* and *S. roeselii* are based on the work of Foissner et al. (1992). Resting cyst morphology is based on Berger (1999) and Foissner et al. (2007).

RESULTS AND DISCUSSION

In 2016, we selected two species of limnic ciliates, *Blepharisma lateritium* and *Stentor roeselii* under laboratory conditions. We tried to follow and describe the structure of resting cysts of both species. Moreover, we succeeded to capture the process of the encystation, precystic stages and young cysts of *B. lateritium* in the samples. Our results are discussed with available data.

Blepharisma lateritium (Ehrenberg, 1831) Stein, 1859

The first morphological changes occurred 2 – 3 days after the beginning of cultivation. During this initial stage, we recognised a significant decrease in movement activity and a change in the shape of the encysting cell. The typical tear drop to lancet shape (markedly elongated in the direction of the longitudinal axis) of the trophic specimen (Fig. 1a) changed into an irregular oval shape of a precystic cell (Fig. 1b). The posterior and anterior parts of the cell were still recognisable. The body length shortened, compared with the size of the trophic cell (90 × 60 – 80 µm vs. 110 – 170 × 70 – 100 µm). Somatic ciliary rows, buccal apparatus and single contractile vacuole on the side of the cell were still recognisable (about 1 – 3 hours after onset of encystation). The contractile vacuole was visible in all precystic cells. The basic description of the precystic stage is in accordance with the work of Repak (1968). However, besides the fundamental changes in the shape and size of the precystic cells, we also observed a relatively atypical phenomenon. A special „paper layer" (Fig. 1b) was created around these encysting cells during the encystation The paper layer was irregular in shape in this stage. Its thickness was about 10 – 20 µm.

The young cysts (about 3 – 4 hours after onset of encystation) (Fig. 1c) were globular, averaging about 60 – 70 µm in diameter in vivo. The majority of the precystic cells and resting cysts of *B. lateritium* occured individually. The „paper layer" was present around each young cyst. Unlike the precystic stage, it is evenly dispersed around the entire cell.

The young cysts still had typical structures of trophic cells (as in the previous case of precystic cells), e. g. contractile vacuole. The contractile vacuole was not visible in all cystic cells. These structures disappeared after a complete encystation (about 12 – 24 hours after onset of encystation). After that time, we distinguished the mature resting cysts in all culture dishes.

The resting cysts, averaging about 50 – 70 µm in diameter in vivo, retained a spherical shape of young cysts. The mature resting cysts (Fig. 1d) of *B. lateritium* had been previously described briefly in the works of, e. g., Foissner et al. (1992). We did not notice any significant differences in size (50 – 70 µm vs. 60 – 80 µm). Mulish & Hausmann (1989) described somewhat larger cysts of *B. undulans*, averaging about 100 – 150 µm.

Repak (1968) distinguished on the basis of size two types of *Blepharisma stoltei* resting cysts – smaller cysts (60 – 75 µm) generally lacking coloration and larger cysts (110 – 140 µm) with a dark brown cystic wall. In our research of *B. lateritium*, we recorded only one type of cyst.

In mature resting cysts, a cystic wall was created. First, we recorded the presence of a semi-transparent layer. It formed a contiguous layer around each resting cyst and likely represented the outer layer – ectocyst. This statement agrees with the definition of ectocyst of species *Blepharisma stoltei* (Repak 1968) and Cavaleiro et al. (2017). Except for the external ectocyst, we recognised endocyst and mesocystic space within the cystic wall. The mesocystic space was present between the ectocyst and endocyst. Its thickness was about 5 – 7.5 µm. The endocyst was very thin, about 1 µm. In some cells the wall of the encysted cell was visible. All layers of the cystic wall were smooth. There was no specific ornamentation on the surface. In some cases, only bacteria occurred along the ectocyst.

The most significant feature of the resting cysts was the escape apparatus known as the plug (Fig. 1d). According to Repak (1968), Foissner (1993), Calvo et al. (2003), Bourland et al. (2017) and others, some species of ciliates break through the plug apparatus of the cystic wall. The specimens become thin in the middle of the cell and eventually squeeze through the plug. The specific conical-shaped plug of *B. lateritium* is located above the cytoplasm of the encysted cell and separated from it by a thin line. It extends beneath the endocyst. The presence of this structure was confirmed by Repak (1968) and Foissner et al. (1992), as well. Besides the escape device, this plug plays another important role as well during the excystation process. The study by Repak (1968) claims that the substances (most likely polysacharids) collected in the plug do not have only a protective function in the resting cysts, but they also serve as the first source of energy during excystation.

Morphologically similar resting cysts with an escape apparatus in the form of a plug are typical, e. g. peritrich ciliate *Opisthonecta henneguyi* (Calvo et al. 2003), oligotrich ciliates *Pelagostrombidium fallax* (Müller 1996; Müller & Wünsch 1999), *Limnostrombidium viride* (Müller & Wünsch 1999) and some representatives of the genus *Strombidium* (Kim et al. 2008). According to Bourland et al. (2017), unique, smooth, flask-shaped resting cysts with a distinct neck-like escape apparatus are characteristic for the ciliate *Urostomides denarius* (Armophorea). Moreover, the resting cysts of *Heterometopus palaeformis* also had a similar escape aperture, however, the resting cysts are ovoidal in shape (Esteban et al. 1995; Bourland et al. 2017).

On the basis of these data, we can assume that the presence of the plug is not only related to a certain group of ciliates, but occurs within taxonomically different groups.

The cytoplasm of trophic specimens is deeply pink-pigmented (Repak 1968; Giese 1973). This pigment calls blepharismin. In contrast, the resting cysts partially or completely lost their coloration during the encystation process. The partial absence of pigment in resting cysts (as opposed to the presence in the trophic cells) was also observed in other species as well. Repak (1968) confirmed the changes of cytoplasm colour during the encystation process of the species *Blepharisma stoltei*. The pigment was present only in the periphery of the cell's cytoplasm. The changes in cytoplasmic staining during the encystation process of *Blepharisma sinuosum* have been described in Cavaleiro et al. (2017). According to this study, the pink coloration was present in all cytoplasmic content in the precystic stages and in young cysts. But, the pigmentation of the cytoplasm of mature cysts was brown. They observed pigment accumulation only toward the plug. Nevertheless, Cavaleiro et al. (2017) assume that blepharismin possibly plays a important role in cyst biology.

The mechanism of pigment extrusion of genus *Blepharisma* is still unknown. However, based on the results of Repak (1968), this pigment extrusion may be the result of ionic changes at the level of the pellicle and cell membrane associated with cytoplasm coagulation during the late stage of the encystation process. Basic cytochemical analyses of *Blepharisma* resting cysts are summarized by Repak (1968) and Mulish & Hausmann (1989). In this case, further cytochemical research is necessary.

Stentor roeselii Ehrenberg, 1835

We recorded the first resting cysts in cultures approximately 6 – 7 days after the beginning of cultivation.

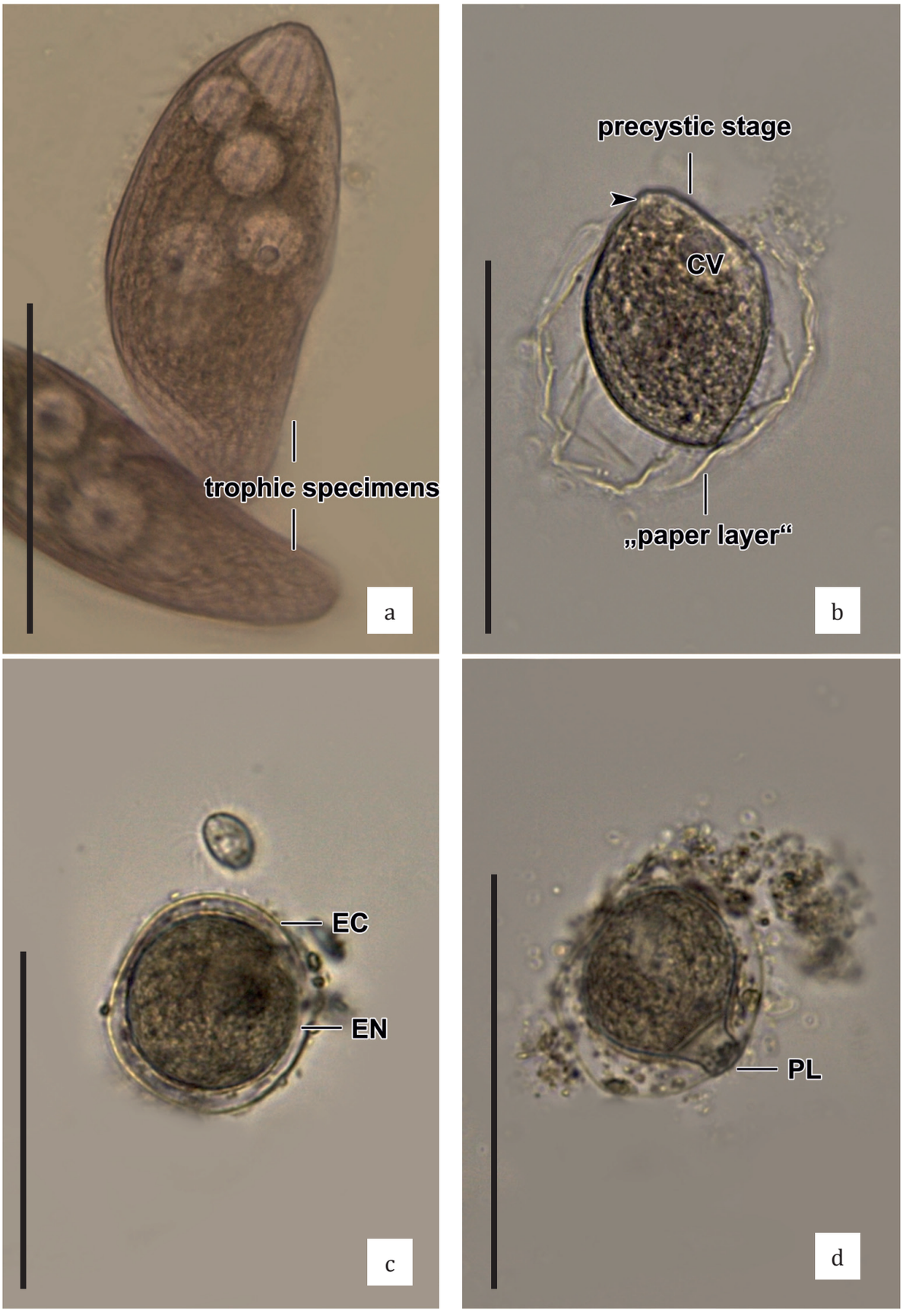

Figure 1. *Blepharisma lateritium* (Ehrenberg, 1831) Stein, 1859.

a – representative trophic specimens in vivo, **b** – precystic stage with specific „paper layer“ around the encysting cell (arrowhead marks the anterior part of cell), **c** – young cyst with the emerging cystic wall, **d** – mature resting cyst with plug. CV – contractile vacuole, EC – ectocyst, EN – endocyst, PL – plug. Scale 100 µm (orig.).

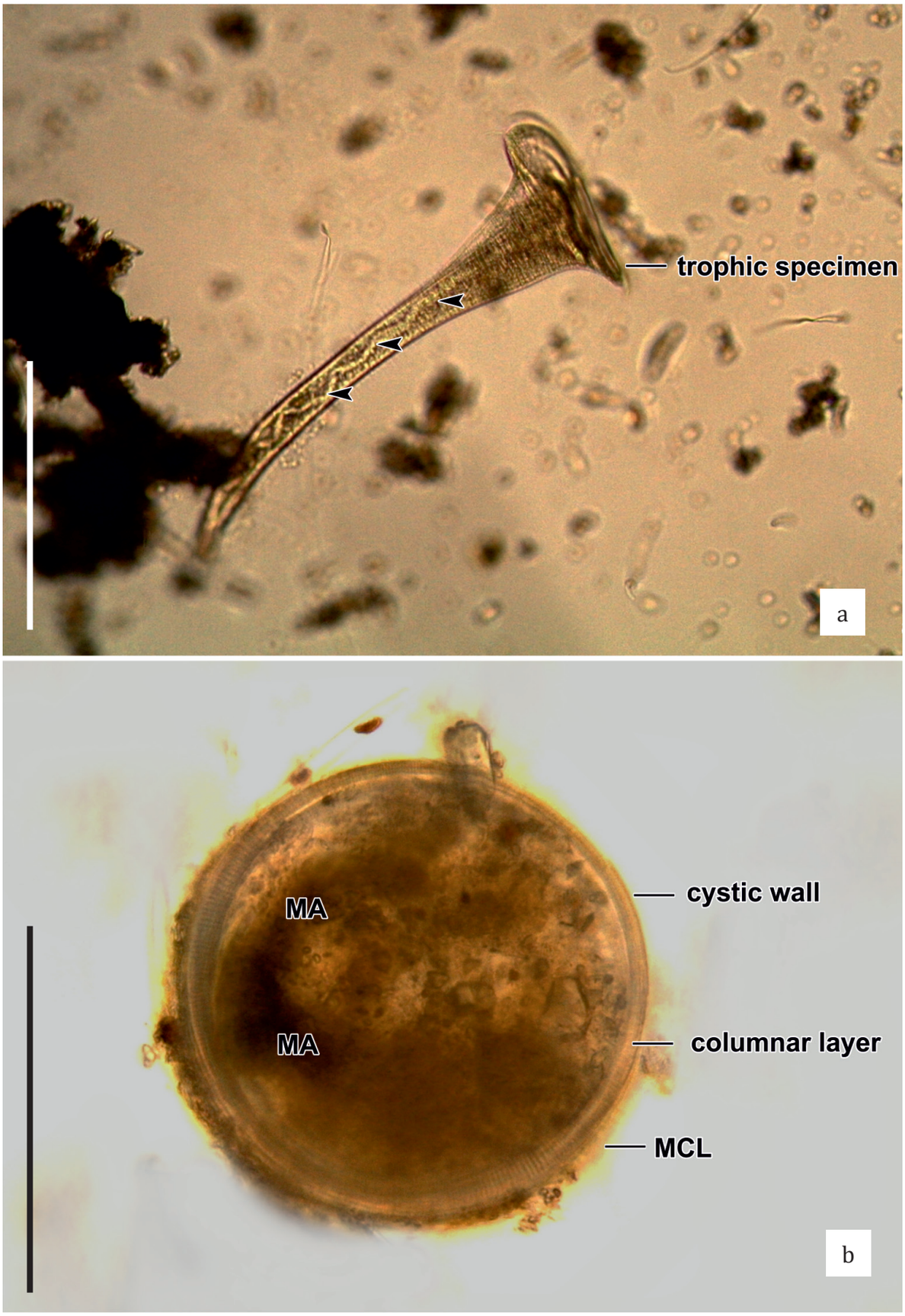

Figure 2. *Stentor roeselii* Ehrenberg, 1835.

a – representative trophic specimen in vivo (arrowheads mark the elongated macronucleus), **b** – resting cyst with unusual pillars (columnar layer) in the form of hem below the cyst surface. MA – macronucleus, MCL – mucous layer. Scales (a) 300 µm, (b) 100 µm (orig.).

All resting cysts were globular (versus horn-shaped trophic specimen), averaging about 120 – 140 µm in diameter in vivo. This represented a significant size decrease from the active specimen dimensions of 500 – 1000 µm (Fig. 2a). In contrast, according to Stein (1867) and Tartar (1961), a bottled shape for the resting cysts of *S. coeruleus* and *S. polymorphus* is characteristic. In contrast, the resting cysts of *S. niger* were described as small, brownish and spherical. Even on the basis of these results, we can assume morphological variability of resting cysts structure within the genus *Stentor*, however, future investigation is still required.

The cytoplasm of *S. roeselii* cysts was much more denser than the cytoplasm of trophic specimens. We identified regularly hooked dark macronuclear mass (Fig. 2b) on the side of the cell (compared to the elongated shape of the macronucleus of the active specimen, it is even rougher but shortened).

The cystic wall had a unique structure (Fig. 2b). It was columnar with many pillars visible in the form of a thin hem just below the cyst's surface(Fig. 2b). The outer layer (most likely ectocyst) appeared quite striated, distally marked by a mucous layer (15 – 20 µm) on the cyst's surface. Similar structure of the cystic wall is characteristic for the resting cysts of the hypotrich species *Caudiholosticha stueberi*. The cystic wall of this species is distally slightly widened with many notched pillars (Berger 2006).

The density of resting cysts in culture was not high. We observed about 1 – 2 cystic cells per 0.5 ml of culture sample. Based on these results, we can assume that the species *S. roeselii* reacts much more slowly than species *B. lateritium* (from the same sample), and the encystation process also takes a longer time. Similarly, the works of Kamiyama (1996) and Chao et al. (2013a, b) confirm the fact that some limnic species of ciliates have a different rate of encystation (e. g. in terms of time). Moreover, it is much harder to induce encystation under laboratory conditions, and the processes of encystation and excystation in natural conditions are bound to certain periods of the year. Seasonal variation of these processes is typical, e. g. for planktonic oligotrich ciliates (Kim & Taniguchi 1995, 1997; Müller & Wünsch 1999; Chao et al. 2013a, b and others).

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