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Puzzle-like Cyst Wall in Centrohelid Heliozoans *Raphidiophrys heterophryoidea* and *Raineriophrys erinaceoides*

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Abstract. The cell body of centrohelid heliozoans is covered with a layer of scales. These scales have species-specific morphology and, since they present in the trophic stage of the cell cycle can be termed "trophic" scales. Several species are known to form cysts; during this process they can produce specific "cyst" scales, different from trophic scales. The present paper describes morphology of cyst scales in two species of centrohelid heliozoans: *Raineriophrys erinaceoides* and *Raphidiophrys heterophryoidea*. The latter species has two types of cyst scales: scales of the first type resemble trophic scales in general structure but, their borders are broad, flattened and not enrolled. Scales of the second type are polygonal and connected to each other by special teeth, forming a single layer organized in a jig-saw puzzle-like manner. In *Raineriophrys erinaceoides* only one type of cyst scale was found. These scales are polygonal and completely different from trophic scales. It is unclear whether these scales form a puzzle-like layer or just overlap each other. Newly excysted individuals keep remnants of cyst scales in their cell coverings and at this stage cyst scales can easily be noted. The morphology of the cyst scales reported here is unlike any other previously reported.

Key words: Centrohelids, cysts, heliozoa, protists, scales, ultrastructure.

INTRODUCTION

Centroplasthelida is a well defined group of axopodiate protists. Most of its representatives possess species-specific scales, covering the cell surface (Mikrjukov 2002, Cavalier-Smith and von der Heyden 2007). Each species has its own unique type of scales or set of different scales types. Individual scale types may be shared by different species, but complete set of scales covering a cell is always species-specific. Encystment under unfavorable conditions, such as starvation, was reported for several centrohelid species: *Acanthocystis turfacea*, *A. penardi*, *Choanocystis aculeata*, *Raineriophrys erinaceoides*, *Pterocystis echinata*, *Polyplacocystis ambigua* (Penard 1904, Rainer 1968). During the encystment, "trophic" scales covering the surface of the trophic cell usually form the outer layer of the cyst wall (Rainer 1968). The fact, that during the process of encystment centrohelids may form additional scales of unusual shape – so-called "cyst scales" was reported by Rainer (1968) for *Acanthocystis penardi* and by Dürrschmidt and Patterson (1987) for *Polyplacocystis*

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ambigua. However, formation and the mode of arrangement of cyst scales was never described; excystment in centrohelids was never observed as well.

Identification and species distinction among centrohelids is mostly based on the scale morphology. Some authors even identify heliozoans on the base of individual scales found in sediments (Wujek 2003, Esteban *et al*. 2012). Thus, precise characterization of all scale types found in every heliozoan species is a necessary part of a species description. In contrast with trophic scales, cyst scales (as well as encystment itself) had very little attention yet. In this paper we describe cysts of two species – *Raineriophrys erinaceoides* and *Raphidiophrys heterophryoidea* – with special attention to their cyst scales. Unique puzzle-like pattern of the arrangement of cyst scales was discovered in *Raphidiophrys heterophryoidea*. To our knowledge, this type of cyst wall has never been observed in protists.

MATERIALS AND METHODS

Water samples contained heliozoans were collected on August 4, 2010 from Lake Nikonovskoe (*Raphidiophrys heterophryoidea* Zlatogursky 2012) and Lake Leshevoe (*Raineriophrys erinaceoides* (Petersen and Hansen 1960) Mikrjukov 2001). Both lakes are located on Valamo Island, Lake Ladoga, North-Western Russia (N 61°23′ E 30°54′). The bottom layer of sediments from the depth of 30–40 cm was collected manually with sterile plastic vials. Samples were transported to the laboratory and inoculated in 90 mm Petri dishes filled with PJ mineral medium (Prescott and James 1955) or with 0.05% cerophyl extract made on PJ medium (Page 1988). Two rice grains were added to each dish to intensify growth of food organisms for heliozoans. Dishes were examined after several weeks of incubation with a phase-contrast inverted Nikon Eclipse TS 100 microscope (40 × lens); heliozoan cells were individually collected by tapering Pasteur pipette, washed in fresh sterile medium and inoculated into fresh 60 mm dishes with 0.05% cerophyl extract to establish clonal cultures. Cultures were stored at +15°C under room light conditions.

Cysts and excysted trophic cells were examined on the glass object slides using Leica DM 2500 microscope (100 × PlanApo lens) equipped with DIC and phase contrast. Intact live specimens were also photographed in the Petri dishes, using phase-contrast inverted Nikon Eclipse TS 100 microscope (40 × lens). All measurements were done on micrographs, using Nikon DF1 camera and accompanying software. In case of cysts, the thickness of the layer of trophic scales was excluded from the cyst diameter because polygonal shape of this layer makes measurements inaccurate. For cyst scales mean diameter was calculated for each individual separately. The total surface areas of cysts were calculated from measurements of the diameters of 6 (for *Raph. heterophryoidea*) and 34 (for *Rain. erinaceoides*) cysts. Measurements of cyst scale area (mean of 69 (for *Raph. heterophryoidea*) and 227 (for *Rain. erinaceoides*) scales) allowed an estimate of the number of scales necessary to cover the exterior of a cyst. The number of cyst scales was counted on light microscopic level, this structures are clearly visible, especially by phase contrast microscopy, which allows us to expect that our counts are close to the total number of cyst scales per individual. For electron microscopy cells were dissolved with Triton X-100 and air-dried as it were described earlier (Zlatogursky 2012).

RESULTS

After several weeks of cultivation spherical cysts were found in cultures of both *Raphidiophrys heterophryoidea* and *Raineriophrys erinaceoides*. In both species diameter of cysts was about 14 μm. The surface of cysts in both species was covered with a layer of trophic scales, forming the outer layer of the cyst wall (Figs 1A, 3A). Cysts were usually attached to the substratum, although occasional floating cysts were found as well. Sometimes empty cyst walls – "shadows" of cysts were seen.

For more detailed observations, cysts were crushed with the cover slip. In these cysts the inner cyst wall, composed of characteristic cyst scales, was clearly visible (Figs 1B, 3B).

In older cultures of both species freshly excysted individuals were seen. It was easy to recognize them due to the presence of the remnants of cyst wall along with usual trophic scales. Otherwise newly excysted individuals looked exactly like usual trophic cells (Figs 1C–E, 3C–E). For *Raph. heterophryoidea* clonal cultures of excysted individuals were obtained; cells in these cultures had trophic scales of usual morphology (Zlatogursky 2012); they never show any scales similar to those seen in cyst stages. Scales and cysts sizes are given in the Table 1.

In *Rain. erinaceoides* from 10 to 88 cyst scales per excysted individual were observed. All scales belonged to a single type – flattened polygonal scales (Fig. 2A). But scales of this basic type were rather polymorphic. Triangle (Fig. 2B), tetragonal (Fig. 2C) and pentagonal (Fig. 2H) scales were found and in many cases the number of angles was difficult to count. Often some of angles or even all of them were rounded and scale became almost O-shaped (Fig. 2G). Another common deviation was elongated or curved shape of cyst scales (Fig. 2F). Even dumbbell-shaped scales were occasionally observed (Fig. 2D).

Each scale had a concave and prominent side. The concave side was smooth, except for one small knob

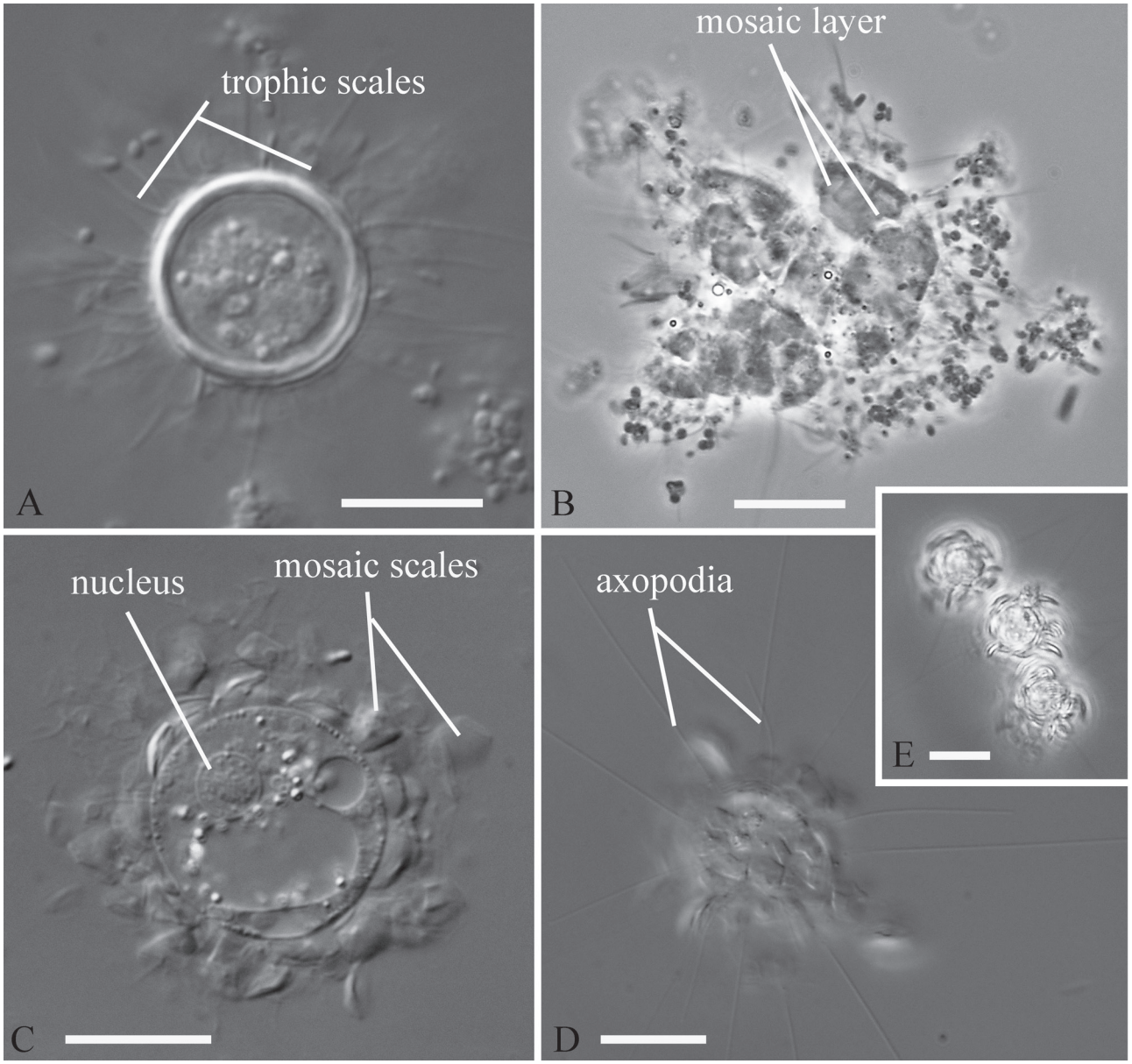

Fig. 1. *Raineriophrys erinaceoides.* Light microscopic images of the cyst (**A**, **B**) and excysted organism (**C**, **D**, **E**). Differential interference contrast (**A**, **C**, **D**) or phase contrast (**B**, **E**). **A –** intact cyst; **B** – a cyst, crushed with a cover slip; **C** – excysted individual, compressed with a cover slip; **D** – excysted individual: view on the cell surface and axopodia; **E –** a group of excysted individuals in a Petri dish. Scale bars: 10 μm.

in the central part (Figs 2B, C). Prominent side usually had a polygonal elevation, which could be either small, occupying only the very center (Fig. 2J) or well-developed, so that only narrow borders remained (Fig. 2F). Sometimes elevation was visible only on one half of the scale or (rarely) was nearly entirely absent. Trophic scales were also present on the encysted cells examined, but certain differences from usual scale morphology were observed in 12 out of 14 individuals, investigated by SEM. In these individuals trophic scales looked more flexible (scales were often deformed) and seemed to have higher shape deviations (Fig. 2I).

In *Raph. heterophryoidea* from 1 to 30 cyst scales per excysted individual were observed. In contrast with

Table 1. Morphological characteristics of cyst scales.

	Parameter	Min	Max	Mean unweighted ± SEM	n	n(i)	Median	Standard deviation	Variation coefficient, %
Raphidiophrys heterophryoidea	Cyst diameter	10.7	22.5	13.7 ± 1.65	6	6	12.6	4.0	29.5
	Type 1 scale length	3.8	4.1	4.0 ± 0.10	48	5	3.9	0.2	5.7
	Type 1 scale area	9.5	12.0	11.0 ± 0.96	25	4	11.3	1.9	17.3
	Type 2 scale length	5.0	7.1	6.2 ± 0.75	62	4	6.3	1.5	24.4
	Type 2 scale area	14.3	28.9	21.3 ± 5.33	69	4	21.0	10.7	50.1
Raineriophrys erinaceoides	Cyst diameter	12.6	16.1	14.2 ± 0.83	34	34	14.1	4.9	34.1
	Scale length	3.3	6.3	4.5 ± 0.62	261	13	4.4	2.2	49.5
	Scale area	7.5	15.3	11.9 ± 1.63	227	14	11.6	6.1	51.3

All length measurements in μm. Abbreviations: SEM – standard error of the mean, Max – maximum, mean – arithmetic mean, Min – minimum, n – number of measurements, and n(i) – number of individuals.

previous species, *Raph. heterophryoidea* had two different types of cyst scales. Scales of the first type somewhat resembled scales of trophic individuals. Both had radial septa, but unlike the trophic scales the cyst scales were surrounded with a broad flattened border (Fig. 4K). All scales of the first type had almost identical oval or slightly polygonal shapes. Scales of the second type were much more complex. As in *Rain. erinaceoides* scales were polygonal with a variable number of angles and well-defined concave and prominent sides (Figs 4A–J). The edge of the concave side bore a row of triangular teeth (Fig. 4A). Some scales of the second type in excysted individuals were still connected with each other. Shape of scales and their teeth fitted together much like the pieces of a jig-saw puzzle or cranial suture lines at the juncture of the plates of a mammalian skull (Fig. 4B). Septa – the key character of the genus *Raphidiophrys* – were also present in scales of a second type. But their septa system was much more complex than in trophic scales. In trophic scales central part was occupied by interconnected compartments divided by septa (Siemensma and Roijackers 1988, Zlatogursky 2012). In the cyst scales of the second type these compartments seemed to be totally separated (Fig. 4C). Beside a central group of compartments there were several concentric rows of smaller additional chambers. Some of the rows were continuous, arranged in irregularly spiral, instead of concentric, manner. Some of the compartment rows were shared between connected scales so that row continued on the adjacent scale (Fig. 4B). Central group of compartments was much more distorted and irregular than in trophic scales and sometimes even had a triangular shape (Fig. 4G). Towards the edge compartments became shorter and finally almost circular. In some compartments very short secondary septa were present (Fig. 4C). Usually, when observed from prominent side, edges of scales of a second type were even, but sometimes parts of the edge were coarsely corrugated (Figs 4F, G). Sometimes interconnected groups of second type scales with several first type scales underneath were observed (Fig. 4D).

DISCUSSION

The present study shows that both *Raphidiophrys heterophryoidea* and *Raineriophrys erinaceoides* are capable of cyst formation. Cyst scales, very different from trophic scales are present in both species. Unlike cyst scales described earlier for *Acanthocystis penardi* (Rainer 1968) and *Polyplacocystis ambigua* (Dürrchmidt and Patterson 1987) in *Raphidiophrys heterophryoidea* cyst scales form a puzzle-like layer; fragments of this layer may be observed in excysted individuals. Light microscopic investigation of crushed cysts and polygonal polymorphic shape of cyst scales also suggests the presence of similar layer in cysts of *Rain. erinaceoides*, however in this species scales have no pronounced teeth forming a puzzle-like connection.

To clarify the situation with the arrangement of cyst scales, I have calculated the number of cyst scales required to form a monolayer on a spherical cyst of given diameter. This calculation show that in *Raphidiophrys heterophryoidea* potential number of cysts

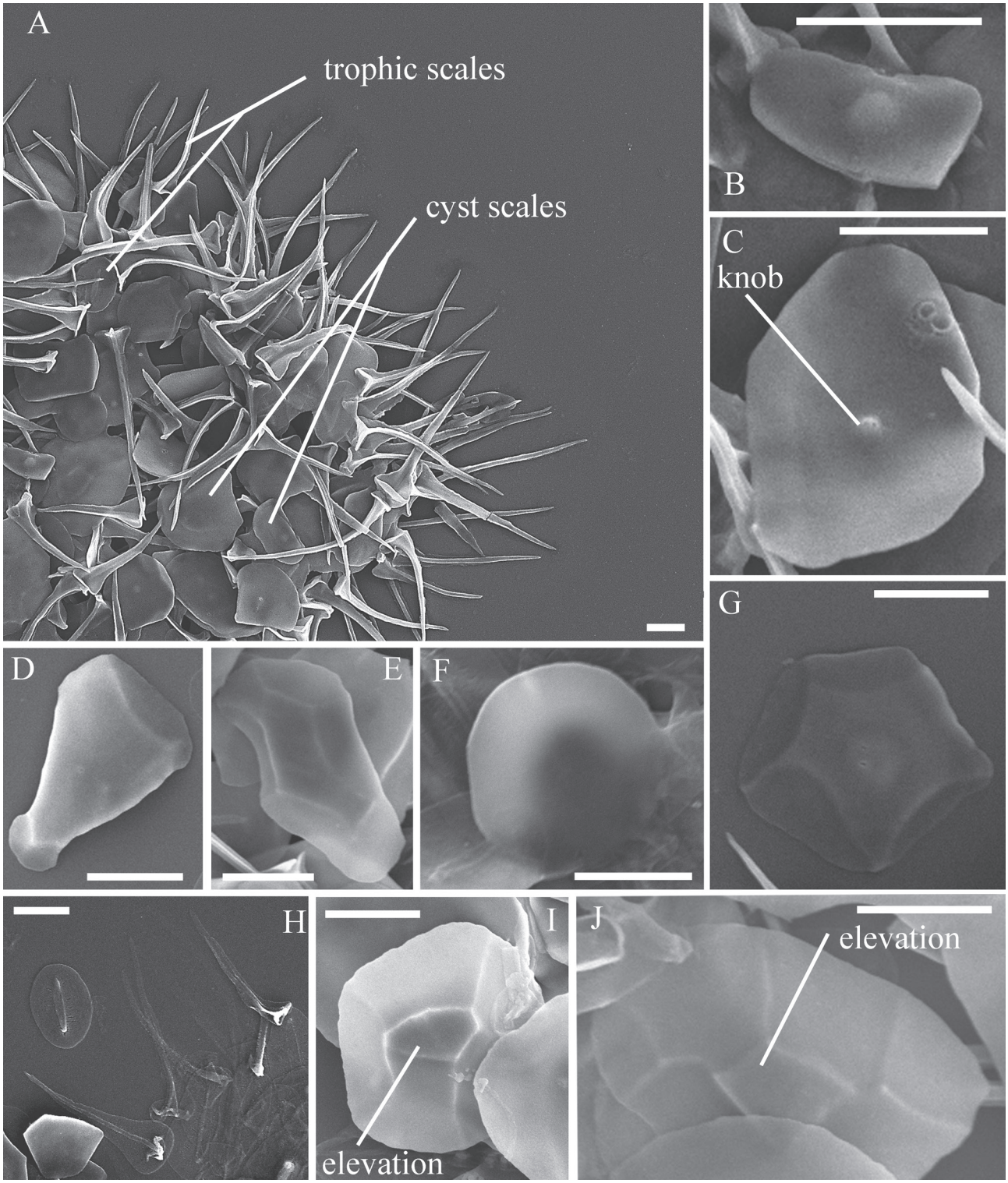

Fig. 2. *Raineriophrys erinaceoides.* Scanning electron micrographs of air-dried excysted individuals. **A –** general view; **B–G**, **I**, **J** – individual cyst scales; **H** – flexible and undersized trophic scales. Scale bars: 2 μm.

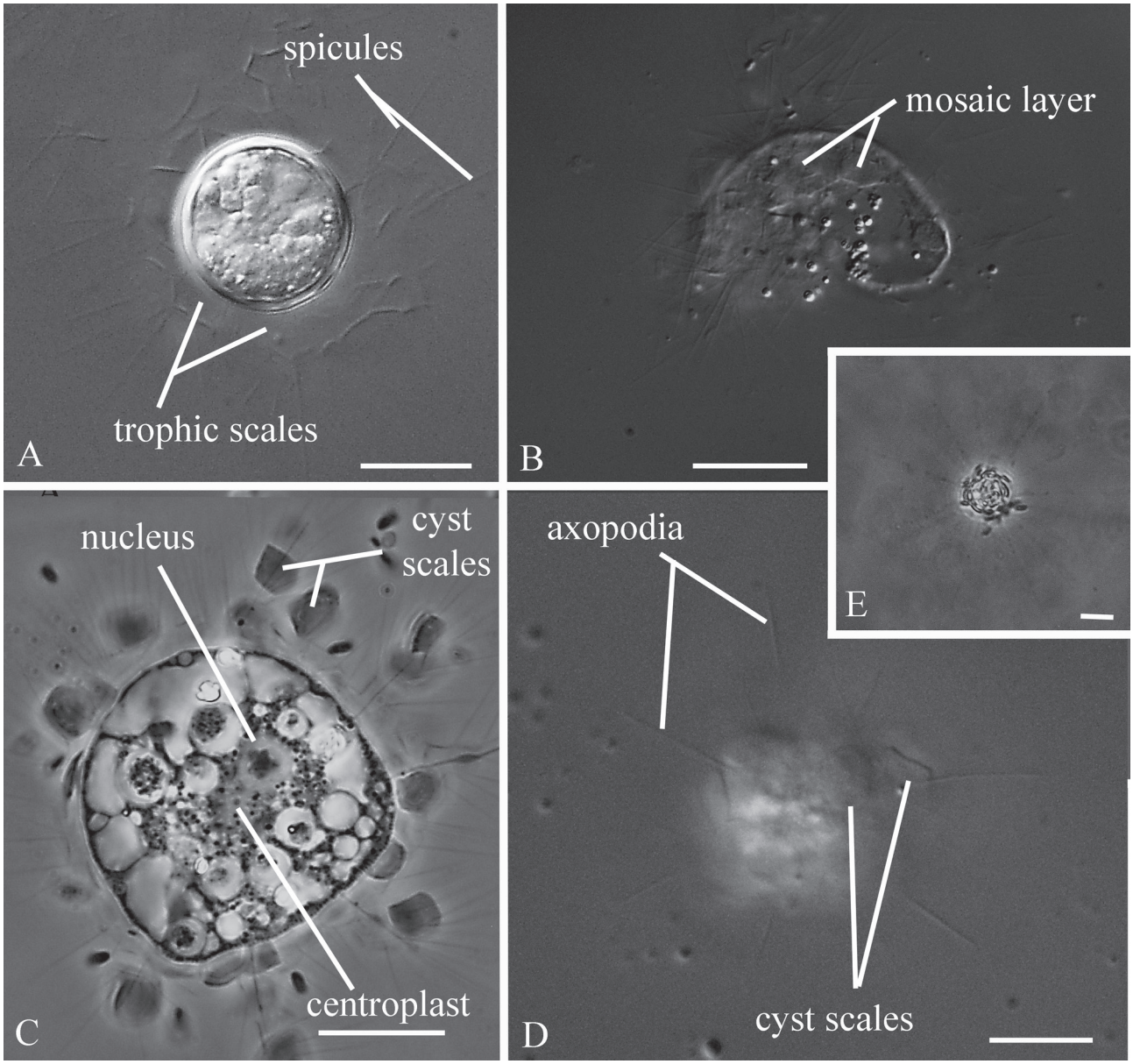

Fig. 3. *Raphidiophrys heterophryoidea*. Light microscopic images of the cyst (**A**, **B**) and excysted organism (**C**, **D**, **E**). Differential interference contrast (**A**, **B**, **D**) and phase contrast (**C**, **E**). **A** – intact cyst; **B** – a cyst, crushed with a cover slip; **C** – excysted individual, compressed with a cover slip; **D** – excysted individual: view on the cell surface and axopodia; **E** – excysted individual in a Petri dish. Scale bars: 10 μm.

scales needed to form a monolayer (30) fits well the observed one (more than 30 scales were never observed). However, in *Raineriophrys erinaceoides* maximal number of cyst scales observed in individual cell (88) was considerably higher than expected one (50). This may indicate that in this species cyst scales partly overlap or that there is more than one layer of cyst scales. This is generally congruent with SEM observations.

Formation of puzzle-like cyst wall is a unique feature and, to my knowledge, it hasn't been described neither for centrohelids nor for any other protists. The formation of somehow similar cyst scales was described for actinophryid heliozoans, particularly for *Actinophrys sol*, but in that case scales were overlapping and did not form a puzzle-like layer (Patterson 1979).

The interesting question is the orientation of scales on the cell surface. In case of *Raph. heterophryoidea*

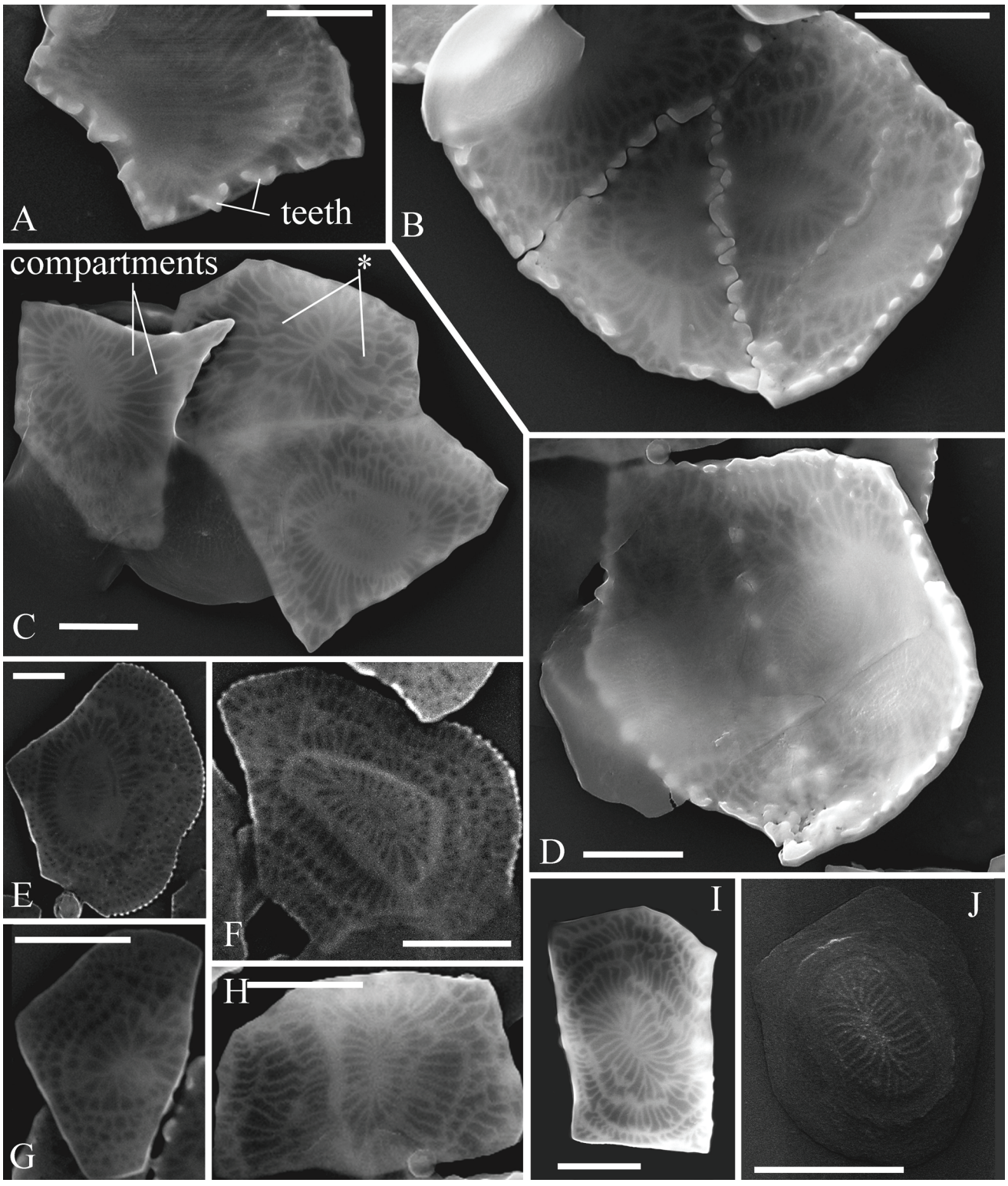

Fig. 4. *Raphidiophrys heterophryoidea*. Scanning electron micrographs of air-dried excysted individuals. **A** – second type cyst scale; view from the concave side; **B** – group of interconnected second type cyst scales; **C** – cyst scales of both types; **D** – group of interconnected second type scales, underlayed with a layer of first type scales; **E–I** – individual second type cyst scales; **J** – individual first type cyst scale. * – secondary septa. Scale bars: 2 μm.

it is clear that concave sides of scales are oriented towards plasmalemma. Otherwise, interconnected scales would not form a spherical layer. Chaotic layer of first type scales, found under a puzzle-like layer (Fig. 4D) suggests that at least some of the first type scales are located closer to the cell surface. In *Rain. erinaceoides* the situation is less obvious, because the way of connection is unknown and both variants of orientation (concave or prominent side to the cell surface) are theoretically possible.

Since number of cyst scales varied between observed excysted individuals and since normal individuals in fresh culture lack cyst scales, it is obvious, that heliozoans gradually shed their cyst scales. Therefore, it is also necessary for them to get rid of old trophic scales from the former cyst wall, because they occupy more peripheral position. New trophic scales must be synthesized *de novo*. One of the possible explanations of flexible and undersized trophic scales in excysted *Rain. erinaceoides* (Fig. 2H) is that we observed underdeveloped scales from a cytoplasm. It is possible, because cells were completely dissolved with the Triton X-100 during specimen preparation. We haven't seen such scales during the investigation of trophic cells by the same method, possibly because scale formation is much less intensive in trophic cells. At the same time, two excysted individuals, studied by SEM had absolutely normal trophic scales along with cyst scales (Fig. 2A), possibly because they excysted only recently. So, active synthesis of new scales had not started yet. Since cyst scales are shed gradually, cyst shadows, observed in cultures are probably not products of excystment, but rather dead cysts with degraded cells inside.

The complexity of a puzzle-like spherical layer in *R. heterophryoidea* suggests that at least final stages of its formation must take place on the spherical surface of a cell. Otherwise the task of fitting of individual scales, formed separately inside the cell would be too difficult. Still, some primary matrixes for future cyst scales may be preformed in the cytoplasm. Since the cyst scales of *R. heterophryoidea* (especially of the first type) are similar with trophic scales it is possible that a trophic scale serves as a matrix for cyst scale formation. In *R. erinaceoides* puzzle-like layer (if present) is less complex and cyst scales do not resemble trophic ones. Therefore, there are no restrictions on cyst scales formation in the cytoplasm.

The present results demonstrate that mechanisms of encystment vary among different species of centrohelids. Individual cyst scales, found in sediments may be identified as a separate species and presence of cyst scales among the trophic ones may also cause a confusion in the identification, even when entire cell is analyzed. Thus, a characterization of cyst scales is necessary for the species description and identification in this group.

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