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ULTRASTRUCTURAL FEATURES OF THE BASAL DINOFLAGELLATE *Ellobiopsis chattoni* (ELLOBIOPSIDAE, ALVEOLATA), PARASITE OF COPEPODS

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ABSTRACT. *Ellobiopsis chattoni* is the type species of the ellobiopsids, an enigmatic lineage of parasitic alveolates that branched between the syndinean dinoflagellates and the perkinsids. We have investigated the ultrastructure of four trophonts from three calanoid copepod hosts collected from the port of Valencia, northwestern Mediterranean Sea. The cell wall showed a thick and homogenous layer and flask-shaped mucocysts that excreted an electron-dense substance that forms the outer layer. The cell wall in the attachment peduncle of *Ellobiopsis* was thicker and with numerous invaginations. The inner section showed numerous longitudinal channels here interpreted as conduits for the transport of host fluids. Trophomere and gonomere were separated by a thin septum with a central pore. Before the mature gonomere detached from the trophomere, the area of junction became undulated. Deficiencies in the fixation of the membrane organelle preclude discussing on other ultrastructural features. To date the ultrastructure of three ellobiopsid genera have been examined. The trophonts of *Ellobiopsis* and *Thalassomyces* showed a high similarity in the cell wall, with characteristic flask-shaped mucocysts. The lack of flask-shaped mucocysts in *Ellobiocystis* and other morphological and ecological differences argue against the monophyly of the ellobiopsids.

Keywords: Apicomplexa, Dinophyceae, perkinsids, Perkinsozoa, Syndiniales, syndinian.

Caracteres ultrastucturales del dinoflagelado basal *Ellobiopsis chattoni* (Ellobiopsidae, Alveolata), un parásito de copépodos

RESUMEN. *Ellobiopsis chattoni* es la especie tipo de los ellobiópsidos, un enigmático linaje de alveolados parásitos que se sitúa entre los dinoflagelados Syndiniales y los perkinsoides. Hemos examinado la ultraestructura de cuatro trofontes que parasitaban tres copépodos calanoides procedentes del puerto de Valencia, Mediterráneo noroccidental. La pared celular presenta una capa gruesa y homogénea con mucocistos con forma de matraz que excretan una substancia electro-densa que forma la capa externa. El pedúnculo de adhesión de *Ellobiopsis* presenta una pared celular más ancha y con numerosas invaginaciones. El pedúnculo en su sección interna muestra numerosos canales longitudinales cuya función se ha interpretado como conductos para el transporte de los fluidos del hospedador. El trofonte y el gonómero están separados por un fino septo con un poro central. Esa región de unión es undulada cuando el gonomero maduro se separe del trofonte. Otros caracteres ultrastructurales no pueden ser descritos debido a deficiencias en la fijación de las membranas de los orgánulos. Hasta ahora se ha examinado la ultrastructura de tres géneros de ellobiopsidos. Los trofontes de *Ellobiopsis* y *Thalassomyces* muestran una gran similitud en su pared celular que presenta el mismo tipo de mucocistos. En contraste, la falta de mucocistos con forma de matraz en *Ellobiocystis*, además de otras diferencias morfológicas y ecológicas, pone en duda el supuesto origen monofilético de los ellobiópsidos.

Palabras clave: Apicomplejos, Dinophyceae, perkinsiode, Perkinsozoa, Syndiniales.

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INTRODUCTION

The alveolates (or Alveolata) are a major lineage of protists divided into three main phyla: ciliates, apicomplexans and dinoflagellates. They share several distinct structural features, the most predominant being a set of flattened membrane-bound vesicles underneath the plasma membrane referred to as alveoli, and mitochondria with ampulliform or tubular cristae (the latter are also shared with a number of other protists) (Lee *et al*., 1985; Cavalier-Smith, 1991). It was these features that led to the first recognition of a relationship between dinoflagellates and ciliates (Corliss, 1975). However, their relationship with Apicomplexa was only recognized following molecular phylogenetic analysis (Gajadhar *et al*., 1991; Wolters, 1991).

Apicomplexans are exclusively animal parasites (exemplified by the malaria parasite *Plasmodium* Marchiafava *et* Celli) (Perkins *et al*., 2000). The dinoflagellates are a diverse and widespread group of protists in aquatic habitats. Their adaptation to a wide range of environments is reflected by tremendous morphological and trophic diversity (Taylor, 1987; Gómez, 2012). The molecular phylogeny has confirmed several morphologically identified lineages that branched between the apicomplexans and ‹core› dinoflagellates or dinokaryotes: Chromerids (*Chromera* Moore *et al.*, *Vitrella* Oborník

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et al.), colpodellids (*Alphamonas* Alexeieff, *Colpodella* Cienkowski, *Voromonas* Cavalier-Smith) are closer to the apicomplexans (Goggin & Barker, 1993; Oborník *et al*., 2012). The perkinsid parasites (*Parvilucifera* Norén, Moestrup *et* Rehnstam-Holm, *Perkinsus* Levine), free-living predators *Oxyrrhis* Dujardin and *Psammosa* Okamoto, Horák *et* Keeling, ellobiopsid parasites (*Ellobiopsis* Caullery, *Thalassomyces* Niezabitowski), parasites of the Marine Alveolate Group I (*Eduboscquella* Coats *et al*., *Ichthyodinium* Hollande *et* Cachon) and Group II (*Amoebophrya* Koeppen, *Hematodinium* Chatton *et* Poisson, *Syndinium* Chatton), and free-living Noctilucales (*Noctiluca* Suriray *ex* Lam., *Spatulodinium* Cachon *et* M. Cachon, *Kofoidinium* Pavill.) are closer to dinokaryotes in the molecular phylogenies (Nóren *et al*., 1999; Saldarriaga *et al*., 2003; Silberman *et al.*, 2004; Skovgaard *et al*., 2005; Gestal *et al*., 2006; Harada *et al*., 2007; Gómez *et al.*, 2009, 2010; Okamoto *et al*., 2012). Ultrastructural studies have been carried out in *Amoebophrya*, *Eduboscquella*, *Hematodinium*, *Ichthyodinium*, *Kofoidinium*, *Noctiluca*, *Oxyrrhis*, *Psammosa* and *Syndinium* (Cachon, 1964; Dodge & Crawford, 1971; Soyer, 1972, 1974; Cachon & Cachon, 1974; Ris & Kubai, 1974; Triemer, 1982; Höhfeld & Melkonian, 1988; Appleton & Vickerman, 1998; Gestal *et al*., 2006; Harada *et al*., 2007; Miller *et al*., 2012; Okamoto *et al*., 2012; Small *et al*., 2012).

Ellobiopsids were first illustrated by Scott (1897) as ectoparasite of copepods in the coasts of Scotland. In the NW Mediterranean Sea, Caullery (1910) described the type species of the ellobiopsids, *Ellobiopsis chattoni* Caullery, as an ectoparasite of a calanoid copepod that he identified as *Calanus helgolandicus* Claus. The lineage of the ellobiopsids contains five genera, of which four (*Ellobiocystis*, *Ellobiopsis*, *Parallobiopsis*, and *Thalassomyces*) are chiefly ectoparasites of pelagic crustaceans, and one monotypic genus *Rhizellobiopsis* parasitizes a benthic polychaetous worm (Shields, 1994). Genera within the family are distinguished by the presence or absence of the attachment peduncle and the number and shape of trophomeres and gonomeres. Gómez-Gutiérrez *et al.* (2010) considered the ellobiopsid *Thalassomyces* as a mesoparasite because it penetrates the external exoskeleton of the euphausiid host. Ultrastructural studies with transmission electron microscopy of ellobiopsids have been carried out in *Thalassomyces* (Galt & Whisler, 1970; Whisler, 1990) and *Ellobiocystis* (Ohtsuka *et al*., 2003).

Copepods are the most abundant animals in the oceans and *Ellobiopsis chattoni* has been reported infecting at least 25 copepod species and even crab larvae (Shields, 1994). The infection is associated with reduction of the host fecundity (Albaina & Irigoien, 2006). The life cycle of *Ellobiopsis* comprises a dispersion phase, a biflagellate spore which settles on an appendage of the host and then becomes ovoid whilst an attachment peduncle develops. When the parasite grows, it becomes transversally septate, with two segments the proximal or trophomere and the distal or gonomere. The gonomere constitutes the reproductive body of the protist where spores are produced (Hovasse, 1952), although exceptionally the spores are also formed in the distal end of the trophomere (Gómez *et al*., 2009). Schweikert and Elbrächter (2006) reported some ultrastructural characteristics of *Ellobiopsis* sp. However, these authors did not report any illustration. This study provides the first transmission electron microscopy (TEM) pictures of the type of the ellobiopsid lineage, *Ellobiopsis chattoni*, based on four trophonts collected from the type locality, NW Mediterranean Sea.

MATERIALS AND METHODS

Specimens were isolated from plankton net samples collected at the port of Valencia, NW Mediterranean Sea (39°N 27*′* 38*″*, 0°W 19*′* 21*″*) in 2011. Live plankton was examined under an inverted microscope Nikon Eclipse T2000, and live copepods infected with *Ellobiopsis* were placed into a vial with cold filtered seawater containing 2% (v:v) glutaraldehyde, and kept in a refrigerator. The fixed specimens were photographed with an Olympus DP71 digital camera in the inverted microscope, place into phosphate buffer (0.1 M, pH 7.2), and post-fixed with 2% osmium tetroxide in phosphate buffer. After rinsing with water, they were sequentially dehydrated in ethanol gradient (30%, 50%, 70% and 96%). Finally, samples were sequentially infiltrated at each step for 2 h in 33% LR-white resin (London Resin Co. Ltd, Basingstoke, UK) in 96% ethanol, 66% LR-white resin in 96% ethanol, 66% LR-white resin in 100% ethanol, 100% LR-white resin in 100% ethanol, and a final step in 100% LR-white resin. The samples were polymerized at 60 ºC for 48 h. Ultrathin sections (60 nm) were finally stained with 2% uranyl acetate prior to viewing by transmission electron microscopy using a JEOL JEM-1010 electron microscope at 60 kV. Images were acquired with a digital camera MegaView III with Olympus Image Analysis Software.

RESULTS

We obtained TEM pictures of sections of four specimens of *Ellobiopsis chattoni* that infected three calanoid copepods tentatively identified as *Paracalanus* sp. (Fig. 1A, 3A) and

Oithona sp. (Fig. 2A). Two trophonts of *Ellobiopsis* from two different hosts were examined in longitudinal sections, including the gonomere and trophomere, and partially the attachment peduncle (Figs 1, 2). Other two trophonts of *Ellobiopsis* attached to the same host were examined in transversal sections (Fig. 3). The four specimens were fixed and conserved together, and they were simultaneously prepared for TEM observations.

The trophont was 110 µm long, and the gonomere was three times larger than the trophomere (Fig. 1A, B). The trophomere showed more electron-dense corpuscles, considered as nuclei, than the gonomere. The nuclei were absent in the proximal part of the trophomere near the peduncle (Fig. 1C). Near the attachment peduncle longitudinal electron-dense structures were observed that are considered channels that harbor materials entering the trophomere (Fig. 1D). The cell wall in the area of the peduncle was thicker (0.5 µm) than in the rest of the trophont. The internal structure was also different, with more irregular surface and traversed by hollow tubes or channels (Fig. 1E-F). These differences with the rest of the trophont cell wall may be related to the two main functions of this area: the adhesion to the host and the suction of the host contents. The rest of the trophont cell wall showed a thick homoge-

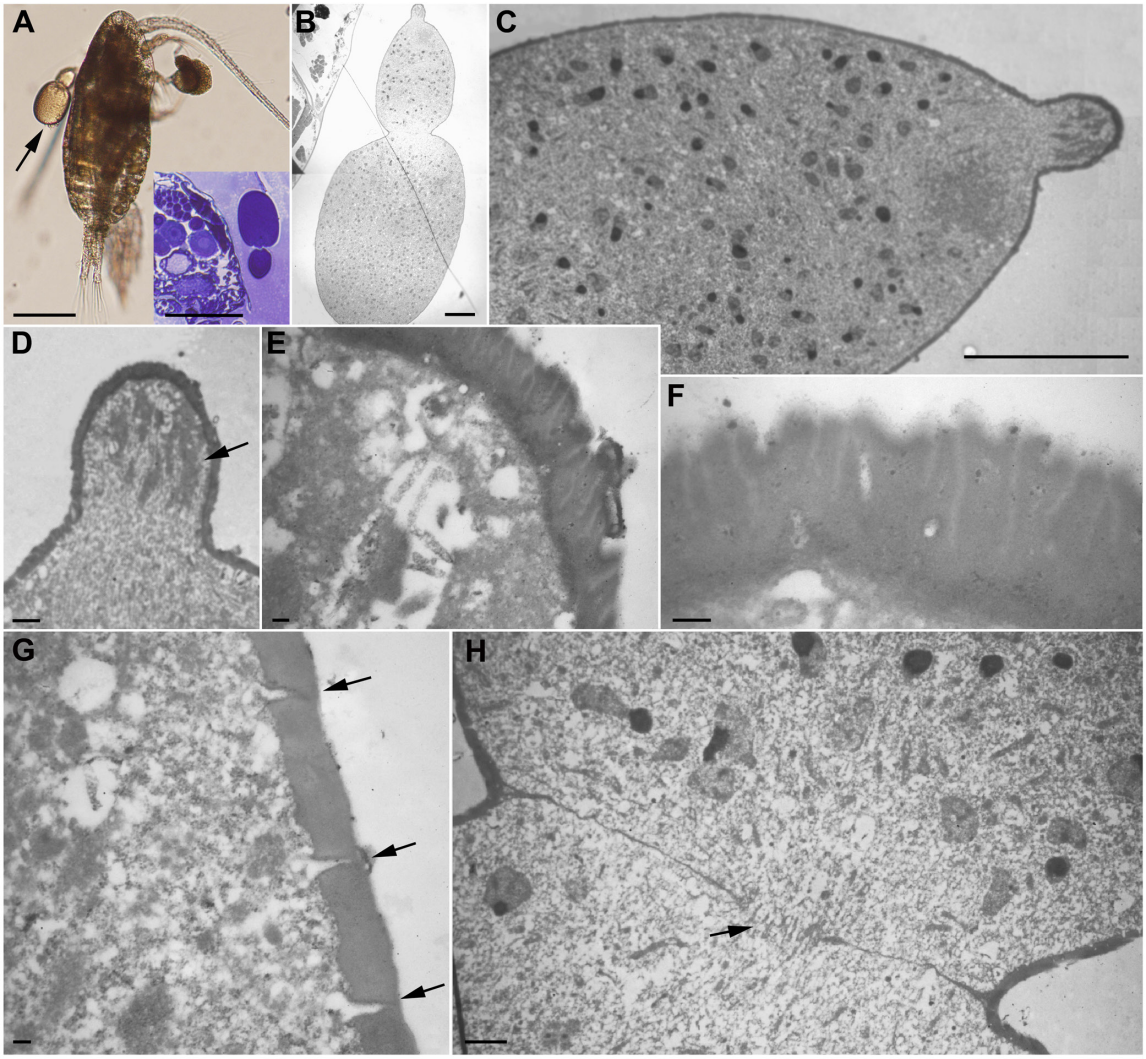

Figure 1. Trophont A of *Ellobiopsis chattoni* as parasite of cf. *Paracalanus* sp. (A). The arrow indicates the examined specimen of *E. chattoni*. (B). Longitudinal section of the trophont. (C). Proximal end of the trophomere and partial peduncle. (D). The arrow points the material entering the peduncle. (E-F). Thicker cell wall in the proximal part of the trophont. (G). Cell wall in the rest of the cell. The arrows point the flask-shaped mucocysts. (H). Septum between the trophomere and gonomere. The arrow points the entering material between the trophomere and gonomere. Scale bars, A-B: 100 µm; C: 10 µm; D, H: 1 µm; E-G: 0.1 µm.

neous layer (~0.3 µm) with flask-shaped bodies inserted perpendicularly in the inner membrane. They were dispersed, often separated 1-1.5 µm (Fig. 1G). The trophomere and gonomere were separated by a thin septum interrupted by a central pore of approximately 2 µm in diameter (Fig. 1H). There were several short, dark linear structures, running perpendicularly to the pore septum (Fig. 1H). This may be interpreted as longitudinal channel or materials entering in the gonomere as observed in the peduncle.

A second trophont of *Ellobiopsis*, located in the antenna of the host, was examined in a longitudinal section (Fig. 2A). The trophomere was pyriform, with a wider distal portion. The gonomere was ovate and wider than the trophomere (Fig. 2B). The distal portion of the gonomere, and more conspicuously in the proximal portion of the gonomere, showed an undulate contour (Fig. 2B, 2E). These observations suggested that the gonomere is under the process of separation from the trophomere, and a new gonomere will emerge after the formation of a new septum in the pyriform trophomere.

As observed in the previous trophont, the cell wall in the proximal part of the trophomere, close to the attachment peduncle, was thicker (0.5-0.7 µm), and the external contour was irregular (Fig. 2C-D). The cell wall showed the flask-shaped mucocysts (Fig. 2F-J). The figures 2F-G showed electron-dense corpuscles at different levels of the mucocyst tubes. This confirms that the flask-shaped bodies are mucocysts that excrete the electron-dense substance of the outer layer. A few large vacuoles were observed in the proximal part of the gonomere. Some large vacuoles showed fibrous or single granule of electron-dense materials of unknown composition and function (Fig. 2K).

Two trophonts infecting the same host were examined in transversal sections (Fig. 3A). The transversal section of first trophont was ellipsoidal (30 µm wide, 35 µm long) (Fig. 3B). The cell wall of the trophont C was slightly thinner (0.2 µm wide) than in the previous trophonts. The outer layer showed discontinuous conspicuous electron-dense films (Fig. 3C). When compared to the previous two trophonts, the cytoplasm

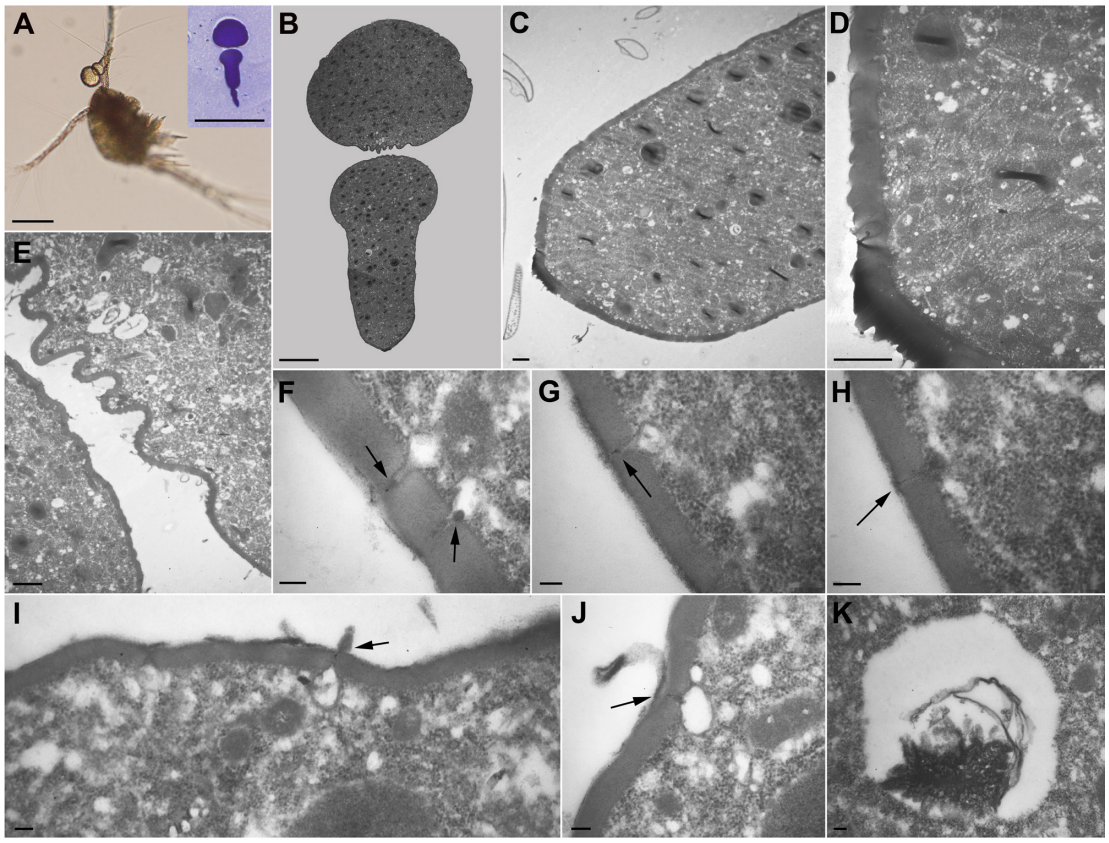

Figure 2. Trophont B of *Ellobiopsis chattoni* as parasite of cf. *Oithona* sp. (A). Copepod and its parasite. (B). Longitudinal section. (C). Proximal part of the trophont. (D). Thicker cell wall. (E). Undulate contour in the junction area between the trophomere and gonomere. (F-J). Flask-shaped mucocysts. The arrows indicate the electron-dense particles excreted by the mucocysts. (K). Unidentified structure inside a vacuole. Scale bars, A: 100 µm; B: 10 µm; C-E: 1 µm; F-K: 0.1 µm.

showed less electron-dense materials. There were large refractive bodies, vacuoles, mainly in the periphery of the cell. There were organelles that showed a peripheral accumulation of electron-dense material that formed a semi- or incomplete circumference (Fig. 3D).

The transversal section of the other trophont was almost round (Fig. 3E). Trophont D showed a cytoplasm with numerous refractive bodies (vacuoles) and a large number of nuclei (1 to 3 µm wide) (Fig. 3H-K). The cell wall showed the electron-dense material (Fig. 3F), and it differed from other trophonts in the more irregular outer contour (Fig. 3G). The nuclei were ellipsoidal and grey stained. The nuclei showed refractive bodies. Electron-dense corpuscles were located in the periphery of the nucleus and in some cases in the center of the nucleus (Fig. 3H-K).

DISCUSSION

Among the parasitic basal dinoflagellates, the trophonts of syndineans, eduboscquellids and perkinsids are endoparasites, often intranuclear parasites. They do not need a special protection against external environmental conditions, except in the motile infective stages. *Ellobiopsis* is an ectoparasite, usually attached to high-exposed areas of the swimming host, and consequently it is subjected to high turbulence, physical damage and the predators of the copepods. This obviously requires a strong attachment system, and a robust cell wall able to protect the organism. *Ellobiopsis* and *Thalassomyces* have a very similar organization of the cell wall and similar flask-shaped mucocysts. As already reported by Galt and Whisler (1970), our results confirm that the flask-shaped mucocysts are excreting a substance that constitutes the electron-dense outer layer (Fig. 2F-J). In addition, the cell wall of *Ellobiopsis* and *Thalassomyces* is thicker than that in the closer alveolate relatives, most of them intracellular parasites that are not exposed to the external conditions.

The alveolates are characterized by the presence of membrane-bound flattened vesicles named alveoli (Cavalier-Smith, 1991). We

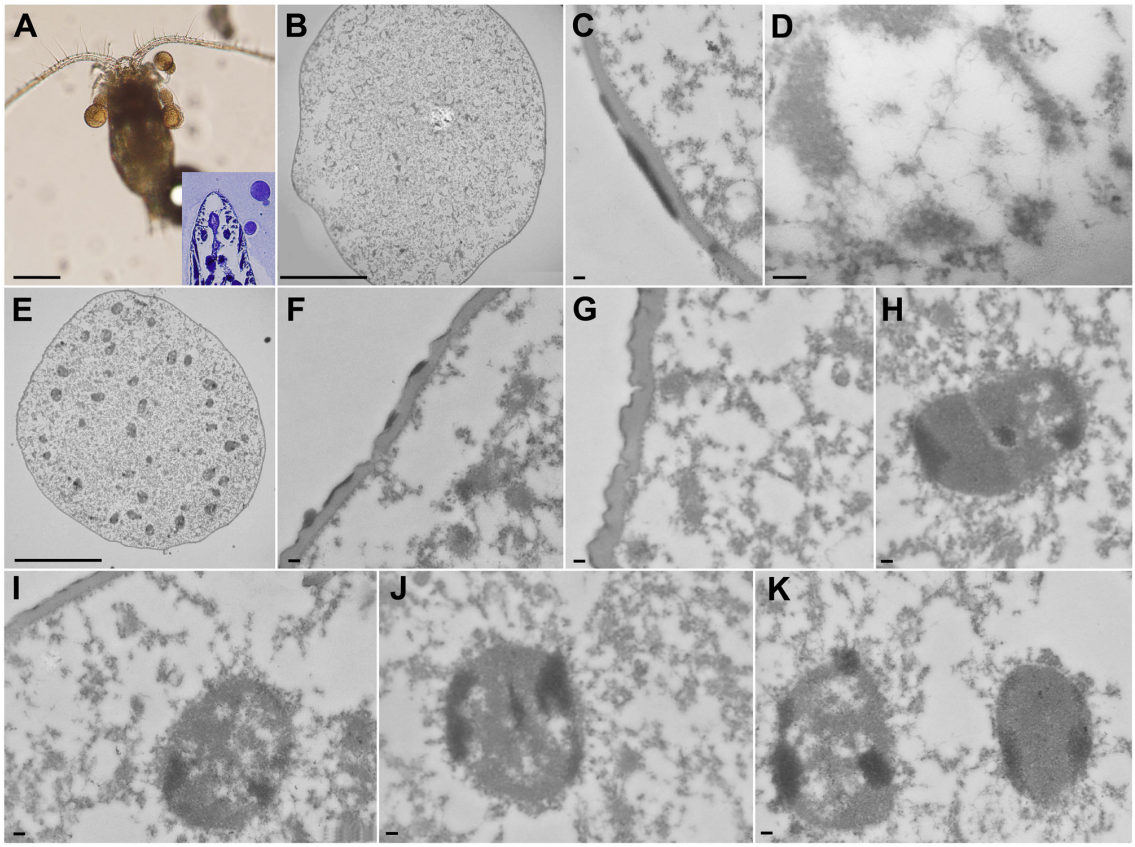

Figure 3. Trophonts C and D of *Ellobiopsis chattoni* as parasite of cf. *Paracalanus* sp. (A). Copepod infected by several trophonts of *E. chattoni*. The larger one is a gonomere of the trophont C (Fig. 3B-D), and the smaller one is the trophont D (Figs 3E-K). B. Transversal section of the trophont. C. Cell wall. Note the electron-dense outer layer. D. Internal structure; E. Transversal section of the trophont D. F-G. Cell wall; H-K. Nuclei. Scale bars, A: 100 µm; B, E: 10 µm; C-D, F-K: 0.1 µm

did not observe the alveoli in the four trophonts of *Ellobiopsis* examined in this study, as also occurred in *Thalassomyces* (Galt & Whisler, 1970). Galt and Whisler (1970: 301) reported "The flagellated spores are bounded by a plasma membrane and lack the elaborate pellicle found in the gonomere and trophomere". In the case of *Noctiluca*, Melkonian and Höhfeld (1988) reported alveoli in some parts of the cell. The presence of alveoli, often referred as amphiesmal vesicles, is a common feature in dinokaryotic dinoflagellates (Netzel & Dürr, 1984). Although in some cases amphiesmal vesicles are not observed (Siano *et al*., 2010). For eduboscquellids (Marine Alveolate Group I), the zoospores of *Eduboscquella* possess cortical alveoli (Harada *et al*., 2007), and *Ichthyodinium* possess flattened alveoli over the external membrane (Gestal *et al*., 2006). For the syndineans (MAGII), the alveoli of *Amoebophrya* were not observed once the parasite entered the host cytoplasm (Miller *et al*., 2012). Miller *et al.* (2012) suggested that the lack of alveoli may facilitate transport of nutrients into the parasite during the intranuclear stage of *Amoebophrya* sp. This is not case of *Ellobiopsis* that is an ectoparasite that absorbs the host nutrients through the attachment peduncle.

The trophont of *Ellobiocystis* was surrounded by a fibrous wall-like structure that differed from that in *Ellobiopsis* and *Thalassomyces*. Bradbury (1994) reported that *Ellobiocystis* may not belong in the Ellobiopsidae, because it is simply attached to the mouth parts and head appendage of its host. There is no connection between the host´s issues and the parasite. In addition to these morphological and ecological differences, the ultrastructure of *Ellobiocystis* differs from *Ellobiopsis* and *Thalassomyces*. We do not have molecular data of *Ellobiocystis* in order to confirm the relationship with the lineage of *Ellobiopsis* and *Thalassomyces* (Silberman *et al*., 2004; Gómez *et al*., 2009). However, the differences of the ultrastructure *Ellobiocystis* argue against about the monophyly of the ellobiopsids.

Ellobiopsis and dinokaryotic dinoflagellate *Oodinium* Chatton resemble in cell shape and the peduncle that in *Oodinium* is attached to the tail of the swimming hosts. *Oodinium* is distantly related to *Ellobiopsis* in its ultrastructure and reproduction (Cachon & Cachon 1971, 1977; Horiguchi & Ohtsuka, 2001). Before the reproduction, *Oodinium* breaks its connection with the host at the peduncle level. This is not the case of *Ellobiopsis*, which trophomere permanently remain attached to the host through the peduncle. The gonomere of *Ellobiopsis* detached from the trophomere after the formation of the immature zoospores (Hovasse, 1952). In this study we observed that the junction between the trophomere and gonomere became undulate when the latter is mature (Fig. 2E). Both parasites, *Oodinium* and *Ellobiopsis*, need a conduct to incorporate the host fluids and a strong attachment peduncle with the host. It seems that both parasites have converged in the ultrastructure of the attachment peduncle. The cell wall of *Ellobiopsis* is thicker in the area of the attachment peduncle. The internal structure is composed of several longitudinal channels instead of a single tube (Fig. 1D). This is the same ultrastructural solution that can be observed in *Oodinium* (Hovasse, 1935; McLean & Nielsen, 1989). The position of the longitudinal channels in *Ellobiopsis* showed a superficial resemble with the longitudinally aligned rod-shaped rhoptries-like vesicles observed in close relatives of *Ellobiopsis* such as *Psammosa*, and it is a typical feature in apicomplexans (Sam-Yellowe, 1996; Okamoto *et al*., 2012). We exclude any evolutionary relationship between the longitudinally aligned channels in the proximal part of *Ellobiopsis* and the rhoptries of the apicomplexans.

A characteristic of *Ellobiopsis* and *Thalassomyces* is the septum between the segments of the cell, trophomere and gonomere(s). The division in septae is a common fungal feature, and for that reason until recently the ellobiopsids were also classified as fungi (Grassé, 1952; Dick, 2001). A further study is required by using a different fixation protocol in order to observe details on the organelle ultrastructure.

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