INTRODUCTION

The gilthead seabream Sparus aurata Linnaeus, 1758, is the most important mariculture species in the Mediterranean Sea, thus constituting one of the most valuable commercial fish for southern Portuguese fish farms. Eight myxosporean species, Kudoa iwatai, Enteromyxum leei (formerly Myxidium leei), Ceratomyxa sparidarum, Polysporoplasma sparis (formerly Leptotheca sparidarum), Sphaerospora sparis (formerly Polysporoplasma sparis), Henneguya sp., Ceratomyxa sp. 1 ex S. aurata, and Ceratomyxa sp. 2 ex S. aurata, have been reported infecting the gilthead seabream in Mediterranean fish farms; some of
these species were associated with increased morbidity and mortality rates (Diamant et al. 1994, 2005, Alvarez-Pellitero et al. 1995, Sitjà-Bobadilla et al. 1995, Bahri et al. 1996, Sakiti et al. 1996, Palenzuela et al. 1999, Sitjà-Bobadilla & Alvarez-Pellitero 2001, Cuesta et al. 2006, Alama-Bermejo et al. 2011). Most of these species are known to occur in southern European sparid-growing farms, but no further knowledge has been acquired concerning other potentially harmful myxosporeans.

During the first parasitological survey for myxosporean parasites infecting sparid fishes in a southern Portuguese fish farm, a new species of Zschokkella Auerbach, 1910 was detected in the gall bladder of Sparus aurata, and is described here based on ultrastructural and molecular data. The genus Zschokkella of the family Myxidiidae Thélohan, 1892 comprises about 68 species, most of which are coelozoic and, less frequently, histozoic, in freshwater and marine fishes worldwide, with a few representatives described from amphibians and reptiles (Lom & Dyková 1992, 2006). Zschokkella spores are ellipsoidal in sutural view and slightly semicircular in valvular view, with rounded or bluntly pointed ends, containing 2 subspherical polar capsules that open subterminally, both to one side (Lom & Dyková 1992, 2006).

The similarity of these morphological characteristics to those displayed by species belonging to the genus Myxidium Bütschli, 1882 makes the distinction difficult between these 2 genera. The application of molecular methodologies to the description of Zschokkella species is therefore crucial, despite the fact that it does not resolve every phylogenetic issue (Fiala 2006, Gunter & Adlard 2008). GenBank provides only 16 Zschokkella sequences of the small subunit ribosomal DNA (SSU rDNA) gene: 5 from freshwater fish hosts (Holzer et al. 2004, Fiala 2006, Bartošová & Fiala 2011) and 11 from marine fish hosts (Palenzuela et al. 2002, Diamant & Palenzuela 2005, Fiala 2006, Freeman et al. 2008, Holzer et al. 2010, Yemmen et al. 2013). Of these, only the sequence of Z. nova has been linked to its correspondent actinosporean stage (Usynskayka 1995). A 17th SSU rDNA sequence termed Z. mugilis Sitjà-Bobadilla & Alvarez-Pellitero, 1993 remains in GenBank; however, this species was recently revised to Ellipsomyxa mugilis (Köie & Karlsbakk 2009), having also been linked to its correspondent actinosporean stage developing in an estuarine polychaete (Rangel et al. 2009, 2012). Overall, the molecular information regarding Myxosporea has revealed the existence of 2 main clades dividing freshwater and marine species (Kent et al. 2000, 2001, Fiala 2006, Bartošová et al. 2009, Holzer et al. 2010), for which myxosporean parasites infecting anadromous hosts, as well as some species of Henneguya, Chloromyxum, Ceratomyxa, Myxobolus, Myxidium, Parvicapsula, and Sphaeromyxa, constitute exceptions (Diamant et al. 2004, Fiala 2006, Rocha et al. 2013). Phylogenetic analyses have also shown that most myxosporeans cluster according to their host tissue tropism rather than by spore morphology or host species (Eszteterbauer 2004, Holzer et al. 2004, Fiala 2006, Bartošová et al. 2009, Fiala & Bartošová 2010), and the genus Zschokkella is no exception (Holzer et al. 2010). Considering this overview, we sought to accurately describe the ultrastructural and phylogenetic features of the parasitic stages found in the gall bladder of Sparus aurata.

MATERIALS AND METHODS

Eighty-nine specimens of the gilthead seabream, Sparus aurata Linnaeus, 1758 (Teleostei, Sparidae) were collected between June 2012 and March 2013 from a fish farm facility on the Alvor estuary, near the Atlantic coast (37° 08' N, 08° 37' W), Portimão, Algarve, Portugal. Upon necropsy, microscopic analysis of several organs and tissues was performed for the detection of myxosporean parasites. Preliminary observations revealed myxosporean plasmodia and spores parasitizing the gall bladder of several specimens. The bile of the parasitized fish, as well as small fragments of the infected gall bladders, were collected and prepared for observation by light microscopy (LM), including differential interference contrast (DIC) optics (Leitz DM RBE, Leica), transmission electron microscopy (TEM), and scanning electron microscopy (SEM), as well as for molecular procedures.

TEM and SEM

For TEM, free spores and plasmodia isolated from the bile were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) at 4°C for 24 h, washed overnight in the buffer at the same temperature, and post-fixed in 2% osmium tetroxide with the same buffer for 3 h at 4°C. Dehydration in an ascending ethanol series and propylene oxide was followed by inclusion in epoxy embedding medium (EPON). Semi-thin sections were stained with methylene blue-Azure II for LM. Ultrathin sections were contrasted with uranyl acetate and lead citrate, observed and photographed using a JEOL 100CXII TEM operated at 60 kV.
For SEM, free spores isolated from the bile were fixed in 5% glutaraldehyde buffered in 0.2 M sodium cacodylate (pH 7.4) at 4°C for 24 h, washed in the same buffer at the same temperature, dehydrated in an ascending ethanol series, critical point dried, coated with a gold-palladium alloy (60%), and observed and photographed in a JSM-6301F SEM operated at 15 kV.

**DNA isolation and PCR amplification**

Free spores and plasmodia obtained from the bile of several specimens were fixed and preserved in absolute ethanol at 4°C. Genomic DNA extraction was performed using a GenElute™ Mammalian Genomic DNA Miniprep Kit, following the manufacturer's instructions. The DNA was stored in 50 µl of TE buffer at −20°C until further use. The SSU rDNA gene was amplified using both universal primers and myxosporean-specific primers: the 5'-end with the primers 18e (5'-CTG GTT GAT CCT GCC AGT-3'; Hillis & Dixon 1991) and R1 (5'-CCT TCC GTC AAT TCC TTT AAG-3'; Azavedo et al. 2009); and the 3'-end with the primers MyxospecF (5'-TTC TGC CCT ATC AAC TTG TTG-3'; Fiala 2006) and 18r (5'-CTA CGG AAA CCT TGT TAC G-3'; Whipps et al. 2003). The sequence region between MyxospecF and R1 overlaps, allowing complete amplification of the SSU rDNA gene. PCRs were performed in 50 µl reactions using 10 pmol of each primer, 10 nmol of each dNTP, 2.5 mM MgCl₂, 5 µl of 10× Taq polymerase buffer (Finzymes), 1.5 units of Taq DNA polymerase, and 3 µl (approximately 100–150 ng) of genomic DNA. The reactions were run on a Hybaid PxE Thermocycler, with initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 45 s, 53°C for 45 s, and 72°C for 90 s. The final elongation step was performed at 72°C for 7 min. Aliquots (5 µl) of the PCR products were electrophoresed through a 1% agarose 1× tris-acetate-EDTA buffer (TAE) gel stained with ethidium bromide. PCR products were purified using a single-step enzymatic cleanup that eliminates unincorporated primers and dNTPs.

**DNA sequencing, and distance and phylogenetic analyses**

The PCR products from different regions of the SSU rDNA gene were sequenced directly. The sequencing reactions were performed using a BigDye Terminator v1.1 from the Applied Biosystems Kit, and were run on an ABI3700 DNA analyzer (Perkin-Elmer, Applied Biosystems).

In order to determine the phylogenetic position of the new Zschokkella species amongst its closest relatives sequenced to date, namely other members of the genus Zschokkella, 58 myxosporean SSU rDNA sequences from GenBank were obtained and analyzed, according to highest similarity score. Tetra-capsuloïdes bryosalmonae and Buddenbrockia plumatellae were selected as outgroup species. The alignment was performed with ClustalW in MEGA 5 software (Tamura et al. 2011), with an opening gap penalty of 10 and a gap extension of 4 for both paired and multiple alignments, resulting in a total of 2380 positions in the final dataset. Subsequent phylogenetic and molecular evolutionary analyses were conducted using MEGA 5. Phylogenetic analyses were performed using maximum parsimony (MP), neighbor-joining (NJ), and maximum likelihood (ML) methodologies. For MP, the close neighbor interchange heuristic option with a search factor of 1 and random initial tree addition of 500 replicates was performed. For NJ, a Kimura 2-parameter substitution model with gamma distribution (shape parameter = 1.4) was performed. For ML, the general time reversible substitution model with 4 gamma-distributed rate variation among sites was performed. All positions with less than 95% site coverage were eliminated from all trees, resulting in a total of 725 positions in the final dataset, and the analysis was run using 500 bootstrap replicates in order to test the robustness of the resulting tree.

After a second alignment only for Zschokkella sequences (resulting in a total of 2194 positions in the final dataset) the distance estimation was carried out in MEGA 5, using the Kimura 2-parameter model distance matrix for transitions and transversions, with all ambiguous positions removed for each sequence pair.

**RESULTS**

Collected and analyzed gilthead seabream did not present external symptoms of infection or disease. Microscopic analysis of 12 different organs revealed the presence of both plasmodia and mature spores floating free in the bile of several specimens. The morphological and ultrastructural features of the mature spores identified the parasite as a member of the genus Zschokkella, according to the classification provided by Lom & Dyková (2006). A new species, named Zschokkella auratis sp. nov., is described...
based on ultrastructural and molecular features, with the following taxonomic position:
Phylum: Myxozoa Grassé, 1970
Class: Myxosporea Bütschli, 1881
Order: Bivalvulida Shulman, 1959
Family: Myxidiidae Thélohan, 1892
Genus: Zschokkella Auerbach, 1910

Zschokkella auratis sp. nov. (Figs. 1 to 14)

**Diagnosis.** Plasmodia and mature spores were observed floating free in the bile (Figs. 1 to 3).

**Description.** Plasmodia were surrounded by a highly irregular cell membrane, entirely covered by fine projections, from which ramified strands of mucus extended, forming glycostyles (Figs. 3 & 4). The surface membrane revealed marked aspects of exocytosis, rather than pinocytic vesicles or canals. The ectoplasmic margin displayed numerous vesicles containing granular material, as well as some dense granules and mitochondria (Fig. 4). The endoplasm further displayed vegetative nuclei, several large vacuoles (Figs. 4 & 5), and numerous disporoblasts simultaneously containing developing and mature spores (Figs. 3, 5 to 7). The earliest sporogenic stages were not observed. The final stages of sporogenesis, namely immature spores, were recognized by their valvogenic, capsulogenic, and sporoplasmogenic cells. In maturing spores, each capsulogenic cell formed a

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Figs. 1−5. Light and transmission electron micrographs of Zschokkella auratis sp. nov. infecting the gall bladder of Sparus auratus. **Fig. 1.** Free fresh mature spores observed in differential interference contrast optics. Notice the surface ridges (arrows) present in the spore wall. **Fig. 2.** Free fresh mature spore, slightly semi-circular in valvular view and containing 2 sub-spherical polar capsules. **Fig. 3.** Semi-thin section of a plasmodium (Pl) displaying irregular periphery (arrows) and containing numerous spores (S). **Fig. 4.** Ultrathin section of a plasmodium (Pl) containing vegetative nuclei (N), numerous segregating vesicles (V) and some large vacuoles (Va), and displaying a highly irregular cell membrane, apparently due to the release of segregated material (arrows). **Fig. 5.** Ultrathin section of a plasmodium (Pl) showing an immature spore (iS) and several mature spores (S) contained within disporoblasts (*).
globular primordium that extended into an external tubule. The valvogenic cells appeared less dense than the mature valves and displayed remnant nuclei (Fig. 6). Mature spores were ellipsoidal in sutural view and slightly semi-circular in valvular view, with rounded ends, measuring $9.5 \pm 0.3$ SD (8.7–10.3) µm in length and $7.1 \pm 0.4$ (6.5–8.0) µm in width/ thickness ($n = 40$; Figs. 1 & 2). Spore wall was thick, composed of 2 symmetrical valves united along a slightly curved suture line (Figs. 7 & 8). DIC and TEM revealed the presence of surface ridges projecting from the spore wall (Figs. 1, 5, 7 & 8). SEM showed each valve bearing 10 to 11 elevated surface ridges, parallel to the suture line, and forming a distinct pattern along the entire spore body (Figs. 12 & 13). Two equal-sized subspherical polar capsules, $3.7 \pm 0.3$ (3.0–4.1) µm long and $3.0 \pm 0.2$ (2.6–3.2) µm wide ($n = 30$), were located subterminally and at the same level within the spores, opening at nearly opposite positions (Figs. 1, 2 & 7). The polar capsules displayed a double-layered wall comprised of an outer electron-dense layer and an inner electron-lucent layer, containing a dense and homogeneous matrix, and along which the polar filament coiled in 4 to 5 turns, terminating in a cap-like structure (Figs. 9 & 10). Upon extrusion, each polar filament exits the
spore body through its respective extrusion pore, located near the suture line (Fig. 11). A large binucleate sporoplasm surrounded the 2 polar capsules and contained numerous electron-dense sporoplasmosomes (Fig. 11). The ultrastructural features observed are represented in a schematic drawing (Fig. 14), providing a better view of the spore’s morphology.

Type host. Gilthead seabream *Sparus aurata* Linnaeus, 1758 (Teleostei, Sparidae).

Type locality. Alvor estuary, near the Atlantic coast (37° 08' N, 08° 37' W), Portimão, Algarve, Portugal.

Site of infection. Gall bladder, floating free in the bile.

Prevalence. 11 specimens out of 89 (12.4%) were infected with *Zschokkella auratis* sp. nov.

Type specimens. One glass slide with semi-thin sections displaying a plasmodium containing several mature spores of the hapantotype was deposited in the Type Slide Collection of the Laboratory of Pathology, at the Interdisciplinary Centre of Marine and Environmental Research, Porto, Portugal, under CIIMAR 201.3.

Etymology. The specific epithet ‘*auratis*’ derives from the specific epithet of the host species.

**Molecular analysis**

The resulting consensus DNA sequence of the SSU rDNA gene, composed of 1996 bp, was deposited in GenBank (accession number KC849425). In total, 58 SSU rDNA sequences, including those with the higher BLAST scores, were aligned with the SSU rDNA sequence obtained for *Zschokkella auratis* sp. nov. There were a total of 725 positions in the final dataset. MP/NJ/ML analyses of the SSU rDNA sequences placed the parasite within the coelozoic clade of the major freshwater clade, clustering together with the sequences of 5 other *Zschokkella* spp. parasitizing the gall bladder and *Myxobolus spirosulcatus* parasitizing the bile ducts of brackish and marine fish hosts, with a bootstrap support value of 85% for MP, 92% for NJ, and 92% for ML. The sequence of *M. spirosulcatus* (AB530261) obtained from the spinal cord of *Seriola quinqueradiata* also clusters within the coelozoic clade of *Zschokkella auratis* sp. nov., to join its other sequence (AB530263) obtained from the bile ducts of *Seriola dumerili* (Yokoyama et al. 2010). The remaining 6 sequences belonging to *Zschokkella* species infecting marine hosts cluster within the major marine clade: 3 together with *Sinuoelinea* sp. forming a clade of species isolated from the excretory system (kidney and urinary bladder); and the other 3 together
with *Ellipsomyxa mugilis* and *E. gobii* forming a clade of species infecting the gall bladder, sister to the marine histozoic clade represented by *Enteroxynx* and *Kudoa* species. The SSU rDNA sequences belonging to freshwater Zschokkella species all cluster within the major freshwater clade: those infecting the gall bladder among the subclades of the coelozoic clade (biliary and urinary tract), and *Zschokkella* sp. AH2003 with other species from the excretory system (kidney and urinary bladder). The histozoic freshwater clade is represented by *Myxobolus* species (Fig. 15).

Pairwise comparisons among the SSU rDNA sequences showed that the 5 *Zschokkella* sequences comprising this subclade are also those presenting a higher percentage of identity to *Zschokkella auratis* sp. nov., i.e. *Zschokkella* sp. PS030203 (93.8%), *Z. solaea* (92.8%), *Z. icterica* (92.7%), *Z. auratis* sp. 1 IF-2006 (92.6%), and *Zschokkella* sp. 3 IF-2006 (91.8%; Table 1).

**DISCUSSION**

The morphology of the myxosporean species described here is consistent with the characteristics defined for the genus *Zschokkella* by Lom & Dyková (2006), differentiated from the genus *Myxidium* based mainly on the position and organization of the polar capsules. Considering that the gilthead seabream inhabits marine and brackish habitats, and further acknowledging that *Zschokkella* species infect both freshwater and marine hosts, all *Zschokkella* records from the gall bladder, freshwater and marine, were considered for morphological comparison. Several studies have reported surface ridges as a reliable feature for morphological comparison (Lom & Dyková 1993, 2006, Bartošová & Fiala 2011); therefore, species with smooth valves were not considered. Among the ca. 68 known *Zschokkella* species, 13 species infecting the gall bladder and presenting spores characterized by the presence of surface ridges are relatively similar to *Z. auratis* sp. nov. but present significantly different morphometrics (Table 2). Morphological comparison between these species reveals the lack of truly differentiating characteristics between *Zschokkella* species, once more acknowledging the importance of molecular based taxonomic studies for this genus.

### Table 1. Comparison of some small subunit ribosomal DNA sequences: percentage of identity (top diagonal) and nucleotide difference (bottom diagonal) obtained by Kimura-2 parameter analysis. The alignment resulted in a total of 2194 positions in the final dataset. For distance estimation, all ambiguous positions were removed for each sequence pair. GenBank accession numbers given in parentheses after the species names.

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Fig. 15. Maximum parsimony tree of the small subunit ribosomal DNA (SSU rDNA) sequence of Zschokkella auratis sp. nov. and other selected myxozoan species. The numbers on the branches are bootstrap confidence levels on 500 replicates corresponding to the maximum parsimony, neighbor-joining, and maximum likelihood (MP/NJ/ML) trees. There were a total of 725 positions in the final dataset. GenBank accession numbers in parentheses given after the species name; scale is given under the tree.
<table>
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<th>Zschokkella spp.</th>
<th>Hosts</th>
<th>Location</th>
<th>SL (µm)</th>
<th>SW (µm)</th>
<th>PCL (µm)</th>
<th>PCW (µm)</th>
<th>nFC</th>
<th>nSR</th>
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<td>3.7 ± 0.3</td>
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<td>10−11</td>
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<td>3.0−3.5</td>
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<td>2.0−3.0</td>
<td>n.g.</td>
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<td><em>Hilsa ilisha</em></td>
<td>Bay of Bengal</td>
<td>12.8</td>
<td>3.4</td>
<td>n.g.</td>
<td>9−12</td>
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<td>13.2−16.6</td>
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<td>10.6</td>
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<td>17.8 ± 1.4</td>
<td>4.2 ± 0.6</td>
<td>n.g.</td>
<td>n.g.</td>
<td>Sarkar (2012)</td>
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</table>

Table 2. Zschokkella spp. characterized by the presence of surface ridges and infecting the gall bladder of freshwater and marine fish. SL: spore length; SW: spore width; PCL: polar capsule length; PCW: polar capsule width; nFC: number of polar filament coils; nSR: number of surface ridges. Measurements are given in µm.
Considering implications of the molecular analysis of *Zschokkella auratis* sp. nov. provided in this paper, it should first be noted that most myxosporean genera are poly-/paraphyletic, and this includes the species of *Zschokkella* (Kent et al. 2001, Freeman et al. 2008), which cluster within different subclades of the main freshwater and marine clades. Our molecular analysis of the SSU rDNA gene showed *Z. auratis* sp. nov. establishing phylogenetic relationships for MP, NJ, and ML in concordance with previously published cladograms (Fiala 2006, Freeman et al. 2008, Holzer et al. 2010). The formation of well-defined coelozoic and histozoic clades for both the freshwater and marine clades, and the fact that *Z. auratis* sp. nov. clusters together with other *Zschokkella* sequences from the gall bladder of marine and brackish fish hosts, as well as *Myxobolus spirofulsulatus* from the bile ducts, strengthens the contention that tissue tropism constitutes an important criterion for the recognition of myxosporean phylogeny (Holzer et al. 2004, 2010, Fiala 2006, Bartošová et al. 2009, Fiala & Bartošová 2010).

Concerning the molecular based division of myxosporeans into the major freshwater and marine clades (Kent et al. 2000, 2001, Fiala 2006, Bartošová et al. 2009, Holzer et al. 2010), we note that the clade comprising *Zschokkella auratis* sp. nov. constitutes yet another exception to this main division. Several studies show that myxosporean parasites infecting anadromous hosts, as well as some species of *Henneguya*, *Chloromyxum*, *Ceratomyxa*, *Myxobolus*, *Myxidium*, *Parvicapsula*, and *Sphaeromyxa*, constitute exceptions to the major division into the freshwater and marine clades (Diamant et al. 2004, Fiala 2006, Azevedo et al. 2009, Rocha et al. 2013). However, 4 of the 6 species comprising the clade containing *Z. auratis* sp. nov. contradict this hypothesis: *Zschokkella* sp. PS030203, *Zschokkella* sp. 3 IF-2006, and *Myxobolus spirofulsulatus* were sequenced from strictly marine fish hosts; and, although *Zschokkella* sp. 1 IF-2006 was sequenced from *Eugeres plumier*, a fish known to inhabit marine, freshwater, and brackish waters, the host specimens were captured from the Caribbean Sea, a clearly marine environment (Fiala 2006). Another possible explanation for the existence of a marine subclade within the major freshwater clade lies in the variability of the SSU rDNA gene regions that are expected to be conserved in myxosporean species. Freeman et al. (2008) considered this to be the reason preventing the design of truly ‘universal’ myxozoan primers. Furthermore, many sequences in GenBank, as well as in other molecular databases, constitute incomplete records or lack more vigorous pruning of the ambiguous regions in primary alignments, thus hindering the true acknowledgment of myxosporean phylogeny. Nevertheless, to assess these possibilities, additional information from the SSU rDNA gene of other myxosporeans, as well as of other genera, would be necessary.

Overall, no significant relationship between spore morphology and phylogenetic grouping was evident. The spore-based taxonomy is rather artificial between *Zschokkella* and *Myxidium*, as well as other genera, with the molecular approach enhancing the already vague boundaries between these genera (Fiala 2006, Lom & Dyková 2006, Freeman et al. 2008). Further research must strive to revaluate the taxonomy and phylogeny of these taxa.

Turning to consider the ultrastructure of the sporogenic development of *Zschokkella auratis* sp. nov., it is assumed that the lack of early sporogenic stages and prevalence of mature spores in the plasmodia indicate a somewhat synchronous development. The ultrastructural aspects of plasmodial development described here are not similar to those usually recognized in coelozoic myxosporeans, viz. variations in size and the differentiation of peripheral projections for nutritional intake (Sitjà-Bobadilla & Alvarez-Pellitero 1993, 2001, Rocha et al. 2011). In the case of *Z. auratis* sp. nov., the irregularity of the plasmodia surface appears to result from the exocytosis of segregated material contained within the numerous cytoplasmic vesicles, rather than from the formation and differentiation of peripheral projections. The contents of the vesicles appear to incorporate the mucus branches, forming the glycostyles that cover the entire surface of the plasmodia. The latter constitute a rather rare feature for myxosporean plasmodia, with a few species exhibiting similar structures (Current 1979, Current et al. 1979, Lom 2004). Curiously, spores of *Henneguya pilosa* display a similar glycostyle-like coating (Azevedo & Matos 2003). The factors interfering with the differentiation of cell coats in plasmodia, however, remain unknown, but may be involved with the physical and chemical strengthening of the cellular membrane towards the host immune response, as well as the physical and biological conditions of the organ of infection.

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