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Ceratomyxa drepanopsettae in the gallbladder of Atlantic halibut, *Hippoglossus hippoglossus*, from the northwest Atlantic Ocean

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Abstract. Trophozoites of *Ceratomyxa drepanopsettae* Averintsev, 1907 (Myxosporea: Ceratomyxidae) containing prominent refractile granules were found in the gallbladders of all but one of eight halibut, the exception being a single juvenile. They ranged in shape and size from globular forms 5–10 μm in diameter, to rounded structures with pseudopodia and one or more processes that were up to 500 μm in length and packed with refractile granules. Some trophozoites were free in the bile, while others were attached to the epithelium of the gallbladder wall by pseudopodia which extended between the microvilli. Many free trophozoites were attached to each other by septate junctions between their pseudopodia. There were small cylindrical papillae on the surface of the trophozoites, and the rounded portions contained two vegetative nuclei, generative cells (some attached by junctions) and, in many cases, feeding vacuoles. During sporogony, a binucleate sporoplasmic cell and the capsulogenic cells of some sporoblasts were engulfed by valvogenic cells before they began to differentiate; whereas other sporoblasts consisted of six cells attached to each other, two being capsulogenic cells containing external tubes, two sporoplasmic cells and two valvogenic cells. There was a septate junction around the opening of the rounded polar capsule of the spore, between the capsulogenic and valvogenic cell. Sporoplasmosomes appeared to form in smooth membraned vesicles, possibly part of the Golgi apparatus. Spores had a thin, delicate membrane, and elongate shell-valves, most of which were asymmetric, and bent or folded. A sporoplasm extended on either side of the distinct, straight suture line, but did not penetrate into the valves.

The myxosporean parasites *Ceratomyxa drepanopsettae* Averintsev, 1907, *Leptotheca* sp., and *Myxidium sphaericum* Thélohan, 1895 have all been reported from the gallbladder of Atlantic halibut from the northwestern Atlantic Ocean (Khan et al. 1986). *C. drepanopsettae* was originally described in plaice, *Pleuronectes platessa,* and the rough dab or American plaice, *Drepanopsetta platessoides* (now *Hippoglossoides platessoides*) by Averintsev (1907, 1908a, 1909). It has since been found in other flatfish, including Atlantic halibut, from several geographical locations (Auerbach 1912, Kudo 1918, Polyanskii 1955, Shulman 1966, Zubchenko 1980, Khan et al. 1986, Wierzbicka 1988, 1990).

We found *C. drepanopsettae* in gallbladders from both wild and captive halibut. *C. drepanopsettae* has caused health problems among Atlantic halibut broodstock according to Dr. G. A. Bristow (pers. comm.). He found that all broodstock at one culture site had the parasite, and one female that had been the major egg producer was in poor condition, had not eaten for 6 months, and had a shrunken gallbladder "completely filled with a white pus which was the parasite. The duct was completely occluded." Averintsev (1908a, 1909) suggested that the formation of a multicellular, differentiated anlage of the spore of *C. drepanopsettae* might indicate a link between protists and metazoans; and there has recently been renewed interest in the Myxozoa, because there is molecular evidence that they are metazoans rather than protists (Smothers et al. 1994). *Ceratomyxa* Thélohan 1892 is one of the oldest and largest genera of the class Myxosporea, but the poor quality of many descriptions makes taxonomic comparisons almost impossible (Sitjà-Bobadilla and Alvarez-Pellitero 1993a).

The principal taxonomic diagnostic has been spore structure, but this can be quite plastic (Smothers et al. 1994). *Ceratomyxa ramosa* Averintsev, 1908, described only in Atlantic halibut, has spores similar to those of *C. drepanopsettae* but can be differentiated by its distinctive trophozoites according to Averintsev (1908c). The trophozoites of *C. drepanopsettae* and *C. ramosa* have only been studied using light microscopy, and only drawings are available (Averintsev 1908a,b,c, 1909, Auerbach 1912, Polyanskii 1955) apart from light micrographs of the trophozoites of *C. drepanopsettae* taken with dark field illumination (Kudo 1918).

According to Lom and Dyková (1992), in myxosporeans of some genera such as *Ceratomyxa* and *Sphaerospora* which have pseudoplasmodia, generative or secondary cells proliferate to form two sporoblasts. Each sporoblast contains two valvogenic cells, which spread around two sporoplasmic and two capsulogenic cells, and adhere to each other by cell junctions similar to desmosomes. Endogenous division to form tertiary cells may also occur.

Descriptions of the sporogenesis of *Ceratomyxa drepanopsettae* have only been carried out by Averintsev (1908a, 1909), illustrated by drawings. He found that most trophozoites had two vegetative and two generative cells, the latter being a macrogametocyte and a microgametocyte, that were unequal in size in many cases. These gametocytes divided, and each macrogametocyte fused with a microgametocyte, and each zygote gave rise to a spore. This does not agree with later studies on myxosporeans, which show that the only sexual process is the fusion of the two nuclei of the sporoplasm while it is in the spore, or soon after emergence (Lom and Dyková 1992).

Averintsev (1908a, 1909) found that the subsequent divisions of the sporoblast were complex and that frequently anomalous spore formation occurred. The zygote divided into two cells, a larger cell which later divided into two to form the valvogenic cells, and a smaller cell that divided again to form the sporoplasmic and capsulogenic anlagen. The valvogenic anlage was separate from the smaller cells in many trophozoites, whereas in most cases the sporoplasmic and capsulogenic cells remained close together. The nucleus of the sporoplasmic anlage divided into two, to form a binucleate sporoplasm, and the capsulogenic anlage divided to form two capsulogenic cells. This is different to the development of the sporoblast of *C. shasta* Noble, 1950 (Yamamoto and Sanders 1979), in which two uninucleate sporoplasmic cells were formed, which fused later in spore development to form a binucleate sporoplasm, and a group of six secondary cells was formed with the two capsulogenic cells and two valvogenic cells. In both *C. shasta* and *C. drepanopsettae* the valvogenic cells enveloped the sporoplasm and capsulogenic cells (Averintsev 1908a, 1909, Yamamoto and Sanders 1979). The two sporoblasts in the trophozoite usually developed asynchronously in *C. drepanopsettae,* and valvogenic cells that had not yet begun to envelop the sporoplasm and capsulogenic cells of the more advanced spore were almost indistinguishable from the generative cells beginning to divide to form the other spore (Averintsev 1908a, 1909).

Free spores have been described by Averintsev (1908a, 1909) and Wierzbicka (1988, 1990), illustrated by drawings and light micrographs.

We decided to study the stages of *C. drepanopsettae* in the gallbladder of Atlantic halibut using electron microscopy, so that they could be compared with those of other myxosporeans. We have included light micrographs to compare with existing work, as well as ultrastructural studies. Also, we wanted to find out how common this parasite was in the Atlantic halibut available to us, and to study the effects on its host.

MATERIALS AND METHODS

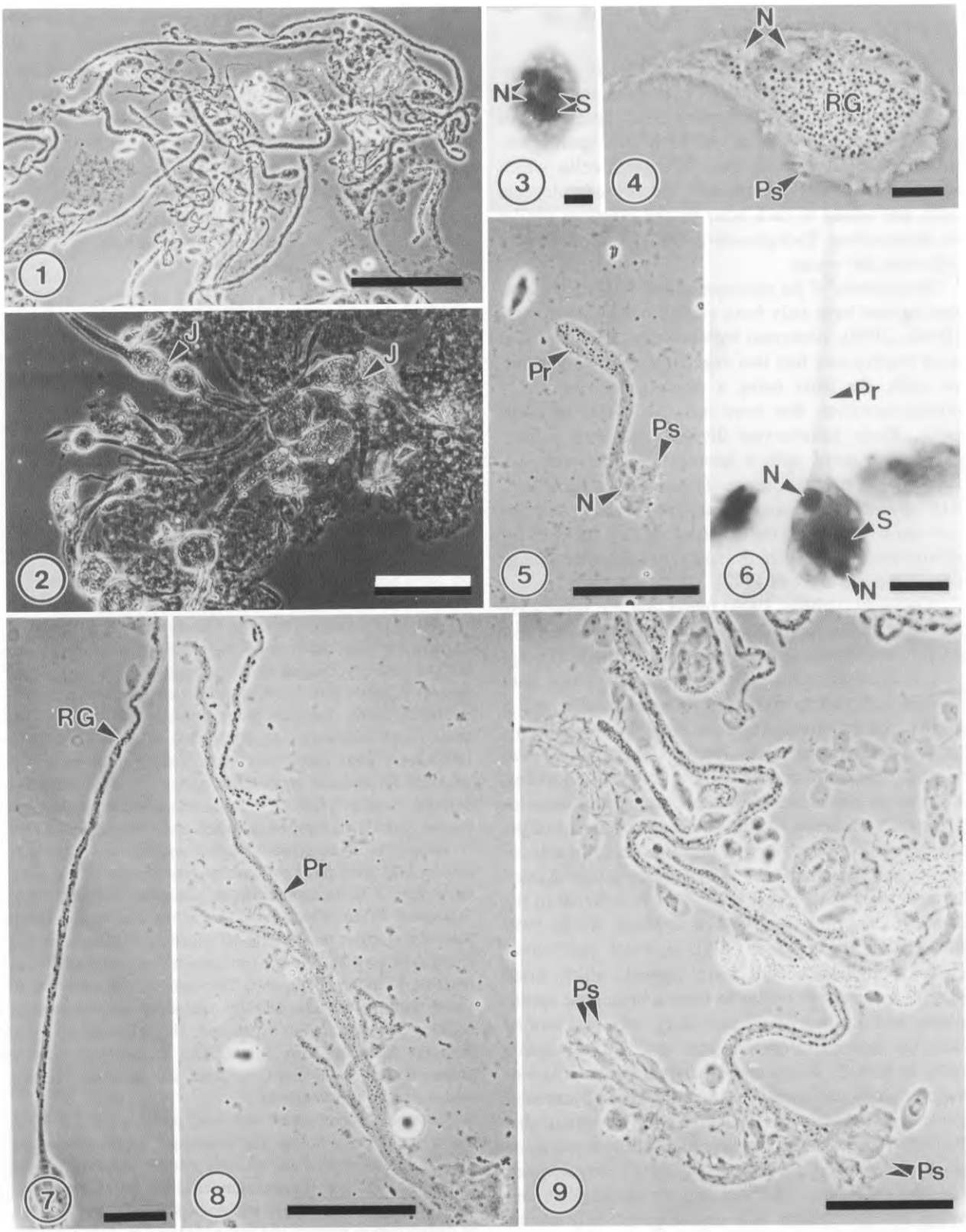
Eight Atlantic halibut, 5 females and 2 males 61 to 113 cm in length and a juvenile 12 cm long, were sampled. One had died in a sea-cage in the Bay of Fundy, off New Brunswick, and was received frozen. Fixed samples were obtained from three broodstock halibut from the St Andrews' Biological Station, New Brunswick; one that was the sole survivor after the rest of the animals died in summer, 1993; one that had been kept in live-holding tanks for about a month in 1994 and one that died in May, 1995. Two halibut, one a juvenile, were brought to Halifax alive from a cruise on the Scotian Banks, off Nova Scotia, and two were brought in live by fishermen at Sambro Fisheries near Halifax, Nova Scotia.

Smears of myxosporea in the bile were examined fresh, or fixed in 1% glutaraldehyde, 4 % formaldehyde in phosphate buffer (1G4F; McDowell 1978). A droplet of bile was placed on a slide coated with 1.5 % agar, then covered with a coverslip (Lom 1969). Samples were studied and photographed using phase microscopy or differential interference contrast (DIC) on a Zeiss photomicroscope. Some smears were also prepared for staining by placing a drop of bile containing parasites fixed in 1G4F on a slide, which was air-dried, then stained with Harris haematoxylin and eosin (Humason 1979).

Samples of myxosporea in the bile and attached to the gallbladder wall were fixed for electron microscopy in 1% glutaraldehyde, 4 % formaldehyde in phosphate buffer (1G4F; McDowell 1978). Myxosporea in the bile were prepared for scanning electron microscopy by pipetting 1G4F containing gallbladder contents into a 10 ml syringe fitted with a 0.45 µm reusable Millipore filter unit. The sample was then passed slowly through the filter and this procedure was repeated for washing and dehydration in ethanol. The filter was removed from the filter unit after dehydration, then it was cut into pieces, critical point dried, mounted on stubs and sputter-coated with gold-palladium.

Pieces of the gallbladder wall were post-fixed in 1.5 % osmium in phosphate buffer. For scanning electron microscopy they were dehydrated in ethanol, critical point dried and sputter coated. For transmission electron microscopy they were dehydrated in acetone and embedded in Epon-Araldite, or dehydrated in ethanol and embedded in LRwhite. Ultrathin sections were stained with uranyl acetate and Reynold's lead citrate. The specimens were viewed and photographed in Hitachi scanning and transmission electron microscopes.

Some fixed samples of *C. drepanopsettae* in the bile were freeze-dried for scanning electron microscopy. They were rinsed in buffer, then filtered onto a 10 µm pore size Poretics



Figs. 1–9. *Ceratomyxa drepanopsettae*. **Fig. 1.** Elongate trophozoites with a variety of shapes and sizes are present in the bile. Squash preparation of material fixed in 1G4F. Bar = 200 μm. **Fig. 2.** The rounded portion of some elongate trophozoites are joined to each other (J) by their pseudopodia. Between the trophozoites are pigmented particulate matter and some crystals. Squash preparation of material fixed in 1G4F. Bar = 60 μm. **Fig. 3.** Small, rounded trophozoite. There are two vegetative or primary nuclei (N), and two secondary cells (S). Squash preparation of material fixed in 1G4F, air-dried then stained with H&E. Bar = 4 μm. **Fig. 4.** Rounded trophozoite. The endoplasm contains prominent refractile granules (RG). Pseudopodia (Ps) extend from the ectoplasmic layer. Two nuclei (N) are surrounded by finely granular cytoplasm. Squash preparation of live material. Bar = 25 μm.

polycarbonate membrane filter, and rinsed in three changes of ethanol (25, 50 and 95 %), followed by three rinses in distilled water. The samples were then plunge frozen in liquid propane at or near liquid nitrogen temperature, and transferred under liquid nitrogen to a Virtis model 10–010 Automatic Freeze-Dryer. The samples were freeze dried overnight, gold sputter coated with an Edwards 306 Coater and examined at 20 kV in a JEOL 35 SEM. Spores were measured from live and fixed material, according to the standards given by Lom and Arthur (1989).

RESULTS

Structure of trophozoites

The bile of all halibut examined, except for the juvenile, was cloudy due to clusters of rounded and elongate trophozoites of *Ceratomyxa drepanopsettae* (Fig. 1). No myxosporeans were observed in a squash preparation of bile from the juvenile halibut. Little movement of live trophozoites was observed. The gallbladder of the dead broodstock halibut that was received in May, 1995 had been fixed whole, and was found to be packed with trophozoites of *C. drepanopsettae*, cell debris and pigmented granular material, presumably bile salts. Some trophozoites were attached to each other by their pseudopodia (Fig. 2). The bile duct did not, however, contain myxosporeans. The trophozoites contained prominent granules which were refractive under phase contrast illumination. In some samples, these granules were brown under normal illumination. There were small, rounded trophozoites 5–10 µm in diameter, some with an elongate process, that had one or two vegetative nuclei and one or two secondary cells (Fig. 3). Larger rounded forms had granular endoplasm, and finely granular ectoplasm with pseudopodia extending from one side (Fig. 4). Most trophozoites had a rounded portion with pseudopodia that contained nuclei, and one or more elongate processes containing granules (Fig. 5). There were two vegetative nuclei and, in small trophozoites, two or three secondary cells (Fig. 6). In some trophozoites, the elongate process was nearly 500 µm long (Fig. 7), in others the process was branched (Fig. 8). Some trophozoites had long, branched pseudopodia (Fig. 9). Two trophozoites were found with nuclei in an oval region in the elongate process.

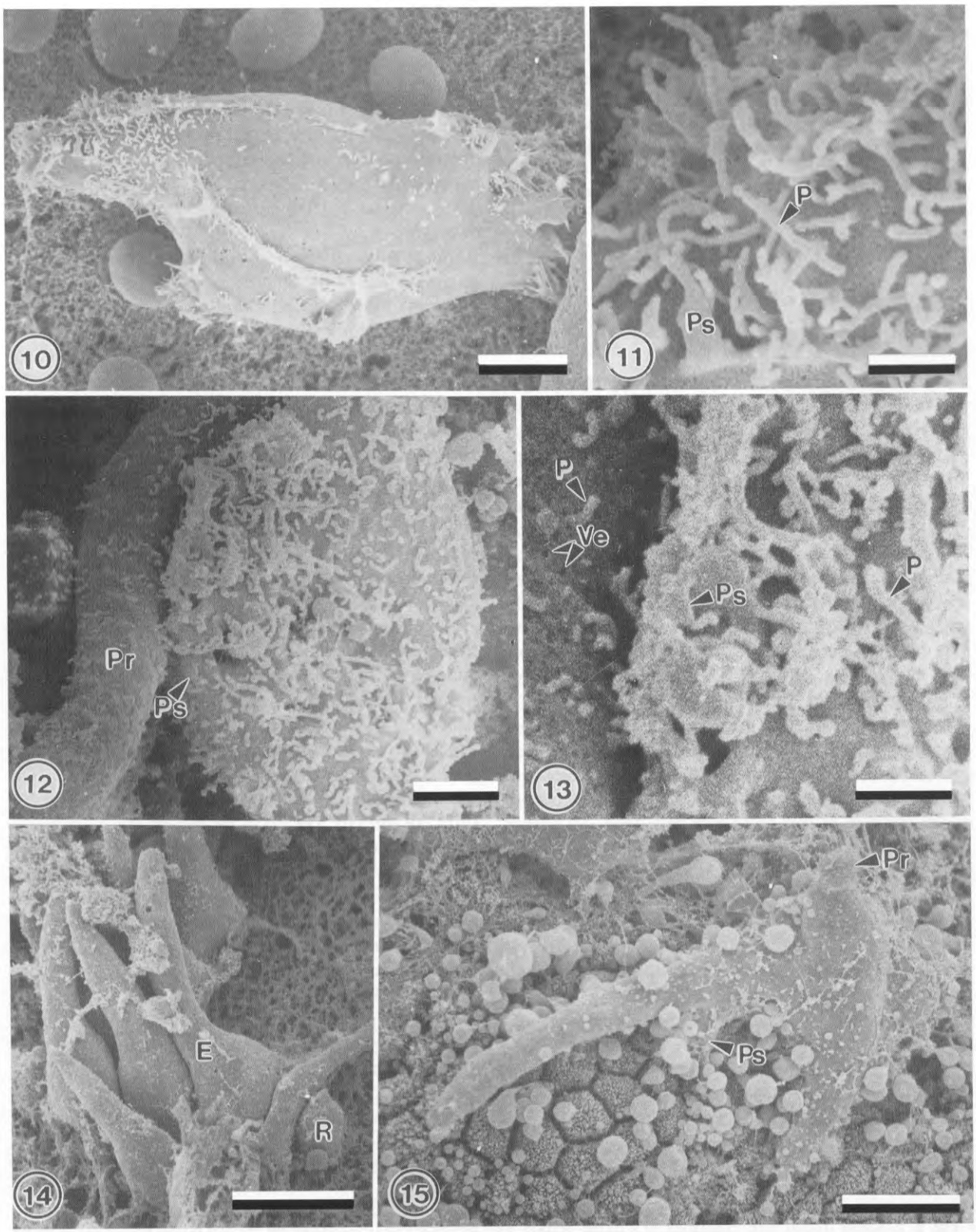
Using scanning electron microscopy, we found that rounded trophozoites had a smooth region, but on most of the surface there were both flattened pseudopodia of varying shapes and sizes and small cylindrical papillae (diameter 0.8–1.4 µm, mean =1.1 µm, standard error = 0.0276) (Figs. 10 and 11). Trophozoites with elongate processes had papillae on the process as well as on the rounded portion, but pseudopodia were only found on the rounded portion (Figs. 12 and 13). Pinocytotic vesicles were seen at the surface of the elongate process (Fig. 13).

Some trophozoites were attached at the rounded end to the surface of the epithelium of the gallbladder wall (Fig. 14) by pseudopodia that penetrated between the microvilli of the epithelial cells (Figs. 15 and 16). The portion of the pseudopodium that extended between the microvilli had dark, filamentous cytoplasm and a dense layer of material at the surface (Fig. 16). The microvilli beneath some attached trophozoites were irregular, and there were regions where groups of detached gallbladder epithelial cells were interspersed with disintegrating trophozoites. Some trophozoites near the gallbladder wall were attached to each other (Fig. 17) by septate junctions with a gap of 23.4 nm (Fig. 18). Many trophozoites had intracellular vacuoles with papillae and coated pinocytotic vesicles (Fig. 17 and insert).

The smallest trophozoites found using electron microscopy were about 10–20 µm in diameter and contained a vegetative nucleus and a secondary or generative cell (Fig. 19). Two vegetative nuclei were visible in many trophozoites. The nuclear membranes of both vegetative nuclei and the nuclei of secondary cells had pores containing dense material except for one region, where they were in contact with the eccentric nucleolus (Figs. 19 and 20). The nucleolar region was more finely granular than the rest of the nucleus. The cytoplasm of the primary cell contained refractile granules, vesicles of varying sizes, and glycogen (Figs. 17–19). There were also elongate tubular mitochondria, some constricted in the middle and dilated at each end, that were especially numerous in the ectoplasm of larger trophozoites (Fig. 17).

Some trophozoites had two secondary cells, many connected by a junction with dense material between the cell membranes, which were 23.9 nm apart (Fig. 20 and insert). The secondary cells had dilated mitochondria with small cristae, some constricted in the centre (Fig. 20); and many had small lobes that extended into the cytoplasm of the primary cell (Fig. 19).

←**Fig. 5.** Elongate trophozoite, consisting of a round portion with pseudopodia (Ps) containing several nuclei (N) of the primary and secondary cells, and an elongate process (Pr) containing many refractive granules. Squash preparation of material fixed in 1G4F. Bar = 100 µm. **Fig. 6.** Small trophozoite with an elongate process (Pr), and two vegetative nuclei (N) and three secondary cells (S) in the rounded portion. Squash preparation of material fixed in 1G4F, air-dried then stained with H&E. Bar = 8 µm. **Fig. 7.** Trophozoite with thin, very elongate process containing refractile granules (RG). Squash preparation of material fixed in 1G4F. Bar = 50 µm. **Fig. 8.** Trophozoite with branching process (Pr). Squash preparation of material fixed in 1G4F. Bar = 100 µm. **Fig. 9.** Trophozoite with long, branching pseudopodia (Ps). Squash preparation of material fixed in 1G4F. Bar = 100 µm.



Figs. 10–15. *Ceratomyxa drepanopsettae*. Scanning electron micrographs(SEM). **Fig. 10.** Rounded trophozoite. Part of the surface is smooth, but there are flattened pseudopodia and small papillae on most of the surface. Sample on Millipore filter. Bar = 5 μm. **Fig. 11.** Part of trophozoite shown in Fig. 10, photographed at a higher magnification. The flattened pseudopodia (Ps) vary in shape and size; whereas the papillae (P) are more constant in width, and some are branched. Sample on Millipore filter. Bar = 1μm. **Fig. 12.** There are papillae on both the rounded portion and elongate process (Pr) of this trophozoite. There are also irregularly shaped pseudopodia (Ps) on the rounded portion. Sample of gallbladder wall. Bar = 2.5 μm.

Sporogenesis and structure of free spores

A binucleate secondary cell was closely associated with another secondary cell in some trophozoites (Fig. 21). In some sporoblasts the binucleate cell could be identified as a sporoplasmic cell that partially surrounded a capsulogenic cell; both were completely surrounded by two valvogenic cells (Fig. 22), which were attached to each other by septate desmosome-like junctions (Fig. 22, insert).

Other binucleate cells, probably valvogenic cells, had dark cytoplasm with regions containing glycogen; there were numerous mitochondria, vesicles and microtubules in the peripheral cytoplasm (Figs. 23–25). They appeared to completely surround one or two tertiary cells, which had a nucleus with an eccentric nucleolus and peripheral, dilated mitochondria (Figs. 23 and 24). Some valvogenic cells were attached to another group of secondary cells by a junction, and many had long extensions around other cells (Fig. 24) or into the cytoplasm of the primary cell (Fig. 26). These extensions contained longitudinally oriented microtubules 20–30 nm in diameter (average 25 nm, n = 15) (Fig. 26). Some tertiary cells had an elongate nucleus with heterogenous chromatin, and a few contained dense, membrane-bound granules similar in appearance to sporoplasmosomes (Fig. 27). There was a nucleolus at each pole of the nuclei of some secondary and tertiary cells.

Many trophozoites contained two sporoblasts with unequal development; and some valvogenic cells were large, with extensive glycogen stores and long extensions into the cytoplasm of the primary cell (Fig. 28). Trophozoites containing developing sporoblasts were larger than those with only one or two secondary cells, and most had one or more elongate processes. These elongate processes contained membrane-bound refractile granules, and specimens from some gallbladders also had osmiophilic droplets which were not membrane-bound, so were probably lipid droplets (Fig. 29). There were coated pinocytotic vesicles both at the surface of the elongate processes, and closely connected with tubules that were continuous with the refractile granules (Figs. 13, 29 and 30). Microtubules were oriented longitudinally in the cytoplasm.

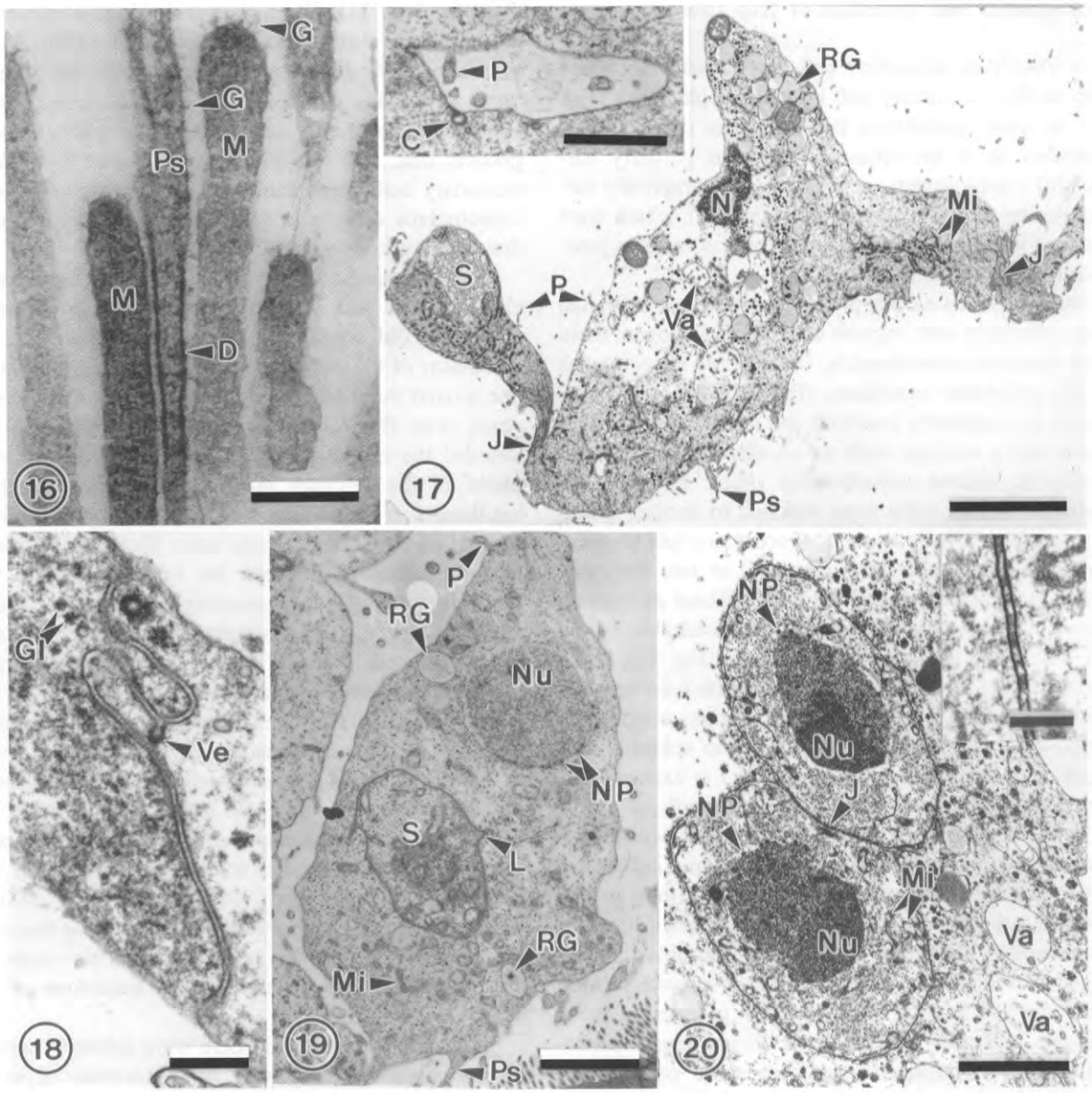
In some trophozoites each sporoblast consisted of a group of six secondary cells, and the capsulogenic cells could be recognised by the presence of an external tube or polar filament primordium. The external tube had a narrow portion with a dense core that was about 330 nm in diameter, and a wider portion about 600 nm in diameter (Fig. 31). The narrower portions of the external tube were surrounded by microtubules (Fig. 31, insert). Each of these capsulogenic cells was closely associated with a secondary cell in which sporoplasmosomes were apparently developing, presumably a sporoplasmic cell; and was partially enveloped by another secondary cell, presumably the valvogenic cell. Some capsulogenic cells in maturing spores were aligned so that they lay side by side (Fig. 32).

The valvogenic cells of the maturing spore were elongate, and had dense fibrous masses in the cytoplasm, but did not develop a thick wall (Fig. 33). The cytoplasm of the valvogenic cells formed a thin envelope around the binucleate sporoplasm and the capsulogenic cells (Fig. 34). The sporoplasm partially surrounded the capsulogenic cells, which had pale cytoplasm. In this specimen, the spores were close together; but there was no junction, and in other trophozoites the spores were separated. There were filaments associated with the junctions between the valvogenic cells. The sporoplasmic cell had extensions into the surrounding vacuolar space, and contained mitochondria with well-developed cristae, extensive whorls of endoplasmic reticulum and granules (Figs. 34 and 35). The granules were of two types; fairly large refractile granules (400–730 nm in diameter, n = 28, mean = 588 nm, standard error = 14.25); and smaller sporoplasmosomes of a more constant size (150–280 nm in diameter, n = 22, mean = 233 nm, standard error = 8.07). There were small, round dense bodies in smooth membraned vesicles (Fig. 35 and insert). Many of the mitochondria were constricted in the middle and dilated at the ends. Ribosomes were closely associated with the outer nuclear membrane and the endoplasmic reticulum, as well as free in the cytoplasm.

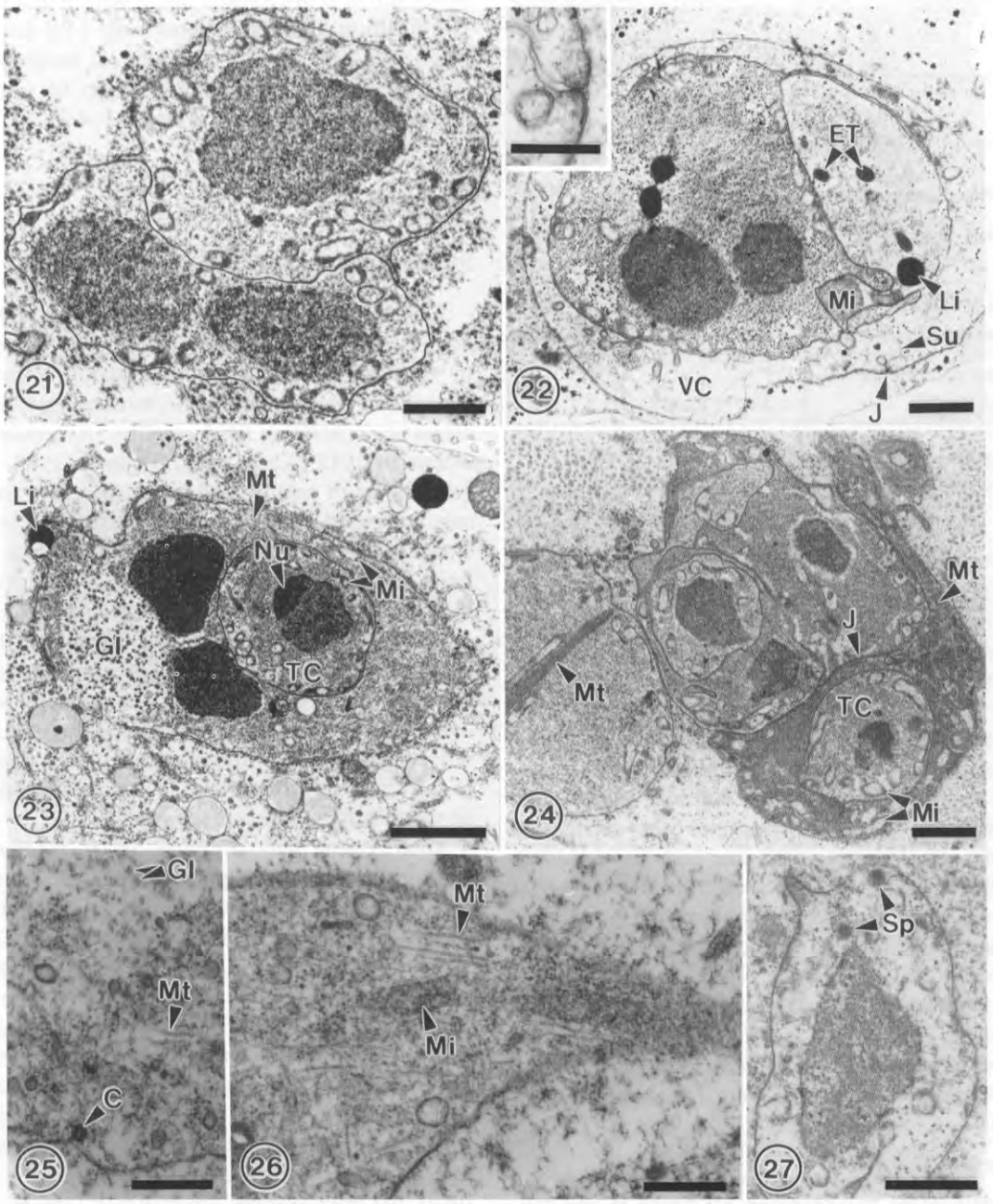
In the capsulogenic cell there were inflated cisternae of endoplasmic reticulum, and processes from the sporoplasm (Fig. 36). There were septate apical desmosome-like junctions 13 nm in width, surrounded by dense material, between the capsulogenic cell and the valvogenic cells (Figs. 36 and 37). The polar capsule was surrounded by an external filamentous layer, and contained a polar filament with 4–5 coils (Fig. 36). There was a proteinaceous cap at its mouth (Fig. 37). The capsulogenic cell contained a nucleus with an eccentric nucleolus (Fig. 38).

Developed spores were found floating freely in the bile fluid (Fig. 39). Many were in groups, attached by the cytoplasmic remains of trophozoites (Fig. 40). The spores contained two round polar capsules opening onto

← **Fig 13.** Part of trophozoite shown in Fig. 12, photographed at a higher magnification. Pinocytotic vesicles (Ve) can be seen at the surface of the elongate process. Sample of gallbladder wall. Ps, pseudopodium; P, cylindrical papilla. Bar = 1 μm. **Fig. 14.** Trophozoites on the epithelial surface of the gallbladder. Some are small and rounded (R), but others have long processes (E). Bar = 20 μm. **Fig. 15.** Trophozoite with short process (Pr), attached to gallbladder wall by pseudopodia (Ps) which extend down among the microvilli of the epithelial surface. Bar = 10 μm.



Figs. 16–20. *Ceratomyxa drepanopsettae.* TEM micrographs. **Fig. 16.** Part of pseudopodium (Ps) of trophozoite between microvilli (M) of epithelial cell at surface of gallbladder wall. There is a glycocalyx (G) on the microvilli and the pseudopodium, and a dense layer (D) at the surface of the pseudopodium where it penetrates between the microvilli. Bar = 500 nm. **Fig. 17.** Group of trophozoites near gallbladder wall attached to each other by convoluted junctions (J). The central trophozoite has a short process containing refractile granules (RG), a nucleus (N) and feeding vacuoles (Va). There are cylindrical papillae (P) on the upper surface of the trophozoites, and pseudopodia (Ps) on the lower surface, which is close to the gallbladder wall. The finely granular ectoplasm contains numerous mitochondria (Mi). The trophozoite at the left is the farthest away from the gallbladder wall, contains a secondary cell (S), and has darker cytoplasm than the other trophozoites. Bar = 5 μm. Fig. 17, insert. Feeding vacuole. Papillae (P) extend into the vacuole, and there are coated pinocytotic vesicles (C) at the periphery. Bar = 1 μm. **Fig. 18.** Detail of junction between trophozoites. The membranes at the surface of each trophozoite are a constant distance apart; and there are septae across the junction. There is a coated vesicle (Ve) at the surface of one trophozoite. (Gl) glycogen rosettes. Bar = 500 nm. **Fig. 19.** Small trophozoite at surface of gallbladder wall. There is a vegetative nucleus, with a nucleolar region (Nu) to one side and nuclear pores (NP) containing dense material at the other, and a secondary cell (S) with small lobes (L) extending into the cytoplasm of the primary cell. There are refractile granules (RG) and mitochondria (Mi) in the cytoplasm of the primary cell, which has small papillae (P) on the surface, and pseudopodia (Ps) extending down to the gallbladder wall. Bar = 3 μm. **Fig. 20.** Two secondary cells of a trophozoite, joined by a junction (J). The nuclear membranes of these cells have pores (NP) except where the eccentric nucleoli (Nu) are next to the membrane. Dilated mitochondria (Mi), some medially constricted, are present in the peripheral cytoplasm. There are feeding vacuoles (Va) in the cytoplasm of the primary cell. Bar = 2 μm. Fig. 20, insert. Junction at higher magnification, showing dense material between the cell membranes. Bar = 300 nm.



Figs. 21–27. *Ceratomyxa drepanopsettae*. TEM micrographs. **Fig. 21.** A binucleate secondary cell partially surrounds another secondary cell. Bar = 1μm. **Fig. 22.** A binucleate sporoplasmic cell almost surrounds a capsulogenic cell containing external tubes (ET). Both cells are completely surrounded by two valvogenic cells (VC) joined by sutures (Su), with a junction (J) at the outer edge. Li – lipid droplet; Mi – mitochondrion. Bar = 2 μm. Fig. 22, insert. Junction at higher magnification, showing septae between the cell membranes, and dense fibrous material beneath them. Bar = 500 nm. **Fig. 23.** A binucleate valvogenic cell, with dark cytoplasm containing a lipid droplet (Li), a region of glycogen (Gl), peripheral mitochondria (Mi) and microtubules (Mt), appears to completely surround a tertiary cell (TC) with peripheral mitochondria (Mi) and a nucleus with an eccentric nucleolus (Nu). Bar = 1.5 μm. **Fig. 24.** A valvogenic cell with dark cytoplasm, and peripheral mitochondria and micro- tubules (Mt), surrounds a tertiary cell (TC) which also has peripheral mitochondria (Mi). It is joined to cells forming the rest of the sporoblast by a junction (J) of constant width. An adjacent cell contains a group of parallel microtubules (Mt), Bar = 2 μm. **Fig. 25.** Periphery of valvogenic cell, with a coated pinocytotic vesicle (C) at the surface. There are vesicles and microtubules (Mt) in the peripheral cytoplasm, and an internal region with glycogen rosettes (Gl). Bar = 500 nm. **Fig. 26.** Extension of valvogenic cell containing parallel microtubules (Mt), mitochondria (Mi) and vesicles. Bar = 500 nm. **Fig. 27.** Tertiary cell with sporoplasmosomes (Sp), and a nucleus containing heterogenous chromatin. Bar = 1 μm.

Table 1. Dimensions of spores of *Ceratomyxa drepanopsettae* (in μm).

	Measurements	n	Range
Thickness of sporoplasm	51.7	23	47.7–67.4
Thickness of valve[a] (from suture line to tip)	39.3	17	34.5–46.9
Length of spore (sutural diameter)	12.6	23	9.9–14.8
Diameter of the polar capsules[b]	4.8	24	4.1–5.8

[a]Total thickness of spore ca. 78.6
[b]The polar filament was distinct, and had 6–8 coils

the anterior surface, surrounded by the pale cytoplasm of the capsulogenic cell. When viewed from the side, up to 7 coils of the polar filament were visible; whereas when viewed from the top or bottom, the spiral nature of the coil was evident (Fig. 41). Rarely, spores were found with 3 valves and 3 polar capsules (Fig. 42). The sporoplasm, which usually contained some refractive granules, extended fairly evenly on either side of the distinct, straight suture line, but did not penetrate into the ends of the spores. Mature spores were surrounded by a thin and delicate membrane, and the valves on either side of the sporoplasm were often bent or folded in on each other, and often asymmetric (Figs. 39 and 43). The total thickness of the spores could not be measured, as no spores were found with two straight valves; and we could not measure the width of the spore, since they always lay flat. The dimensions of fresh spores are given in Table 1. Fixed spores were found to be slightly smaller, having a thickness of about 64.7 μm.

Myxidium sphaericum

The only other myxosporean we found in the gallbladders of the Atlantic halibut was *Myxidium sphaericum*, which occurred in small numbers in only one halibut. Most of the small rounded trophozoites were 16–18 μm in diameter, and many were found in groups. The fusiform, S-shaped spores were 15.9 μm (n = 20, range 13.6–18.5) × 8.5 μm (n = 18, range 6.2–11.1); and the polar capsules were 4.4 μm (n = 18, range 3.1–5.7) × 3.4 μm (n = 19, range 3.1–4.3).

DISCUSSION

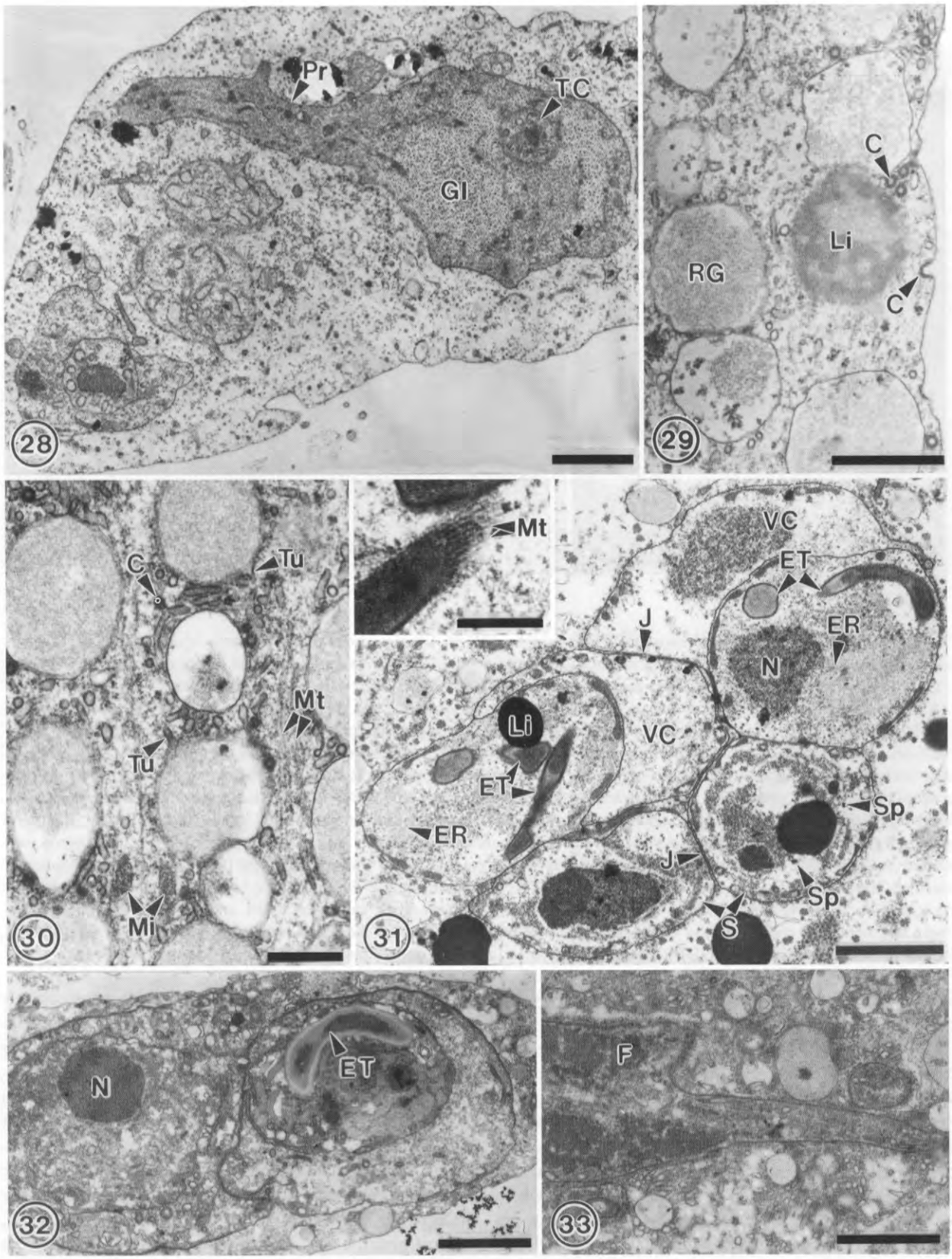
Structure of trophozoites

The structure of the trophozoites of *Ceratomyxa drepanopsettae* in this account agrees with that described by other authors using light microscopy in that the trophozoites are variable in shape, most being elongate and disporous, containing large granules (Averintsev 1908b, Auerbach 1912, Kudo 1918, Shulman 1966). The region devoid of papillae and pseudopodia in some of the small rounded trophozoites is presumably where an elongate process might develop. Averintsev (1908b) found trophozoites in the bile that were long and thread-like, up to 700 μm long, like those we described.

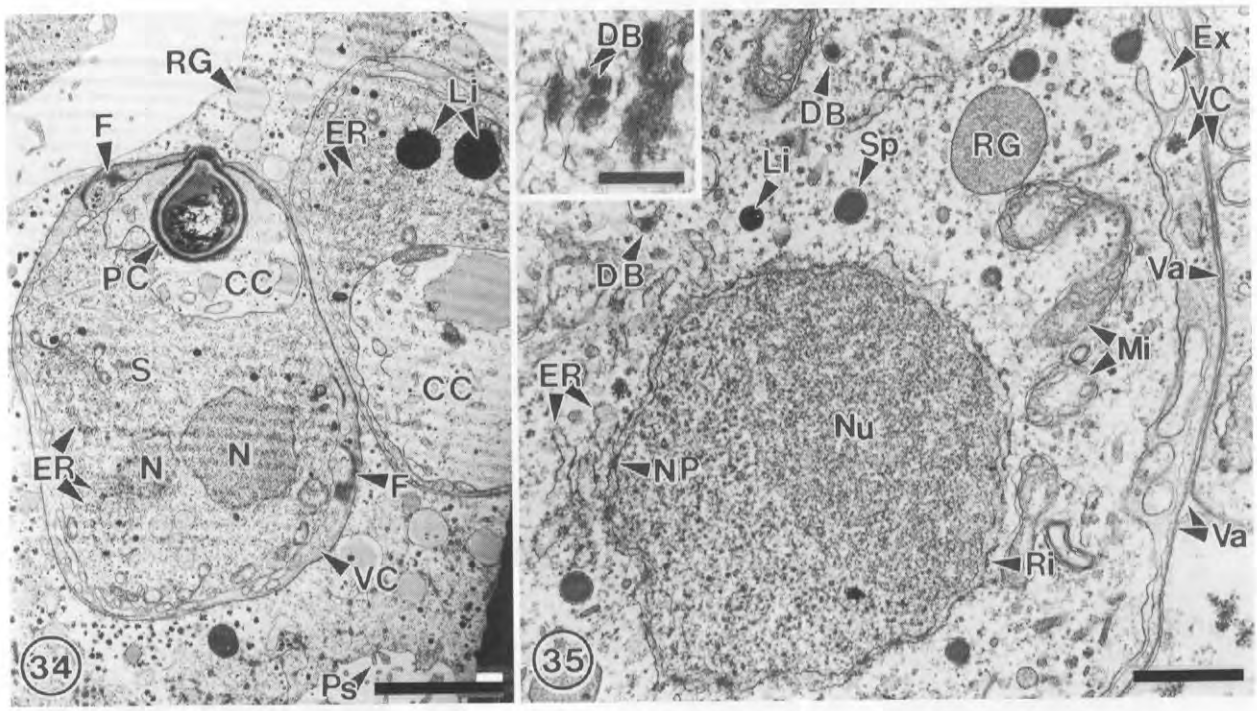
Kudo (1918) showed light micrographs of large distinctive granules in the elongate process; but this author did not mention if the granules were coloured or refractive, like those in our account. Thélohan (1895) described granules of different sizes, colour and degrees of refraction in different species, features which could be valuable for taxonomic purposes.

Sporogenesis may normally occur in *Ceratomyxa* trophozoites with long processes containing refractile granules, because the latter might be a food reserve. Diamant and Paperna (1989) suggested that refractile granules resulted from the coalescing of pinocytotic vesicles. In *C. drepanopsettae* the pinocytotic vesicles appeared to coalesce with long tubules, which were continuous with the granules; so these tubules may form part of a transport mechanism for nutrients that are obtained from the bile by pinocytosis and stored in the granules. Glycogen appears to be the main energy source in myxosporeans (Desportes and Théodoridès 1982), but lipid droplets are common (Shulman 1966). Trophozoites of *C. drepanopsettae* from different halibut varied in the amount of pigmentation of the refractile granules and in their glycogen and lipid content; so

→ **Figs. 28–33.** *Ceratomyxa drepanopsettae*. TEM micrographs. **Fig. 28.** Trophozoite containing two sporoblasts. One is more advanced, and has a large valvogenic cell containing a tertiary cell (TC). The valvogenic cell has dark cytoplasm, a long process (Pr) and large stores of glycogen (Gl). The other sporoblast consists of a group of secondary cells, including a cell with light cytoplasm which has partially enveloped another cell. Bar = 3 μm. **Fig. 29.** Surface of elongate process of trophozoite, containing refractile granules (RG); a more osmiophilic droplet which is not membrane-bound, therefore probably lipid (Li) and small vesicles, including coated pinocytotic vesicles (C). Bar = 1 μm. **Fig. 30.** Part of elongate process of trophozoite showing tubules (Tu) continuous with refractile granules and with a coated vesicle (C); microtubules (Mt) and mitochondria (Mi). Bar = 500 nm. **Fig. 31.** Sporoblast. Two capsulogenic cells contain portions of the external tube (ET), the narrower parts with a dense core. One capsulogenic cell contains a lipid droplet (Li), the other a nucleus (N), and both contain dilated cisternae of endoplasmic reticulum (ER). Each is partially surrounded by a presumed valvogenic cell (VC), and the other two cells are presumably sporoplasmic cells (S). One of the latter contains small dense bodies surrounded by smooth membranes that might be developing



sporoplasmosomes (Sp). There are junctions (J) containing dense material between the pair of valvogenic cells, and the pair of sporoplasmic cells. Each capsulogenic cell is closely associated with its adjacent sporoplasmic cell and enveloping valvogenic cell; there is no gap junction in between. Bar = 2 μm. Fig. 31, insert. Detail of narrow part of external tube surrounded by microtubules (Mt). Bar = 500 nm. **Fig. 32.** Two capsulogenic cells, one sectioned through the nucleus (N) and one containing portions of the external tube (ET), lie adjacent to each other in the sporoblast. Bar = 3 μm. **Fig. 33.** Elongate process of valvogenic cell in developing spore. Dense fibrous material (F) has been laid down in the cytoplasm. Bar = 1.5 μm.



Figs. 34–35. *Ceratomyxa drepanopsettae*. TEM micrographs. **Fig. 34.** Cross-sections of two maturing spores in the rounded portion of an elongate trophozoite. There are numerous refractile granules (RG) in the process of the trophozoite, and pseudopodia (Ps) extend from the base of the rounded portion. A capsulogenic cell (CC) with pale cytoplasm contains a polar capsule (PC), and is partly surrounded by the sporoplasm (S). The sporoplasm has two nuclei (N), cisternae of endoplasmic reticulum (ER) and lipid droplets (Li). Both the sporoplasm and the capsulogenic cells are enveloped by a thin layer of cytoplasm of the valvogenic cell (VC). Fibrous material (F) surrounds the junctions between the two valvogenic cells of the spore. Bar = 4 μm. **Fig. 35.** Sporoplasm of maturing spore. The outer membrane of the nuclear envelope is studded with ribosomes (Ri), and there is dense material in the nuclear pores (NP). The eccentric nucleolus (Nu) is more finely and evenly granular than the rest of the nucleus. There are numerous membrane-bound sporoplasmosomes (Sp) with a dense core, surrounded by a halo, in the cytoplasm; and small, rounded, dense bodies (DB) in vesicles with smooth membranes. There are also many mitochondria (Mi), some constricted in the middle; endoplasmic reticulum (ER); refractile granules (RG) and lipid droplets (Li). Parts of the endoplasmic reticulum have attached ribosomes. There are extensions (Ex) of the cytoplasm into the space between the sporoplasm and the cytoplasm of the valvogenic cell (VC). The outer membrane of the valvogenic cell is thickened, and there is a vacuole (Va) between this membrane and that of the valvogenic cell of the adjacent spore, and the surrounding primary cell. Bar = 800 nm. Fig. 35, insert. There are dense bodies (DB), some rounded, in vesicles with smooth membranes. Bar = 400 nm.

these food stores appeared to be influenced by the condition of the host.

Thélohan (1895) and Shulman (1966) described the trophozoites of most myxosporeans, including some species of *Ceratomyxa,* as irregular in shape, with pseudopodia formed anywhere on the surface. However, many *Ceratomyxa* and *Leptotheca* species have pseudopodia only on the enlarged end of the plasmodia, and not on the opposite pointed end (Shulman 1966), which we have termed an elongate process. Some move actively, with the rounded portion anterior (Thélohan 1895); however, Averintsev (1908b) found, as we did, that the trophozoites of *C. drepanopsettae* were not actively motile.

Averintsev (1908c) described groups of disporous trophozoites of *C. ramosa* in which the pseudopodia were joined together, as described in *C. drepanopsettae* in our study. The presence in *C. drepanopsettae* of junctions between trophozoites and between generative cells as well as in spores may be an additional indication of the metazoan affinities of myxosporeans. These junctions did not have associated supporting filaments like those of the desmosome-like junctions in the spores (Desportes and Théodoridès 1982, Azevedo et al. 1989, Sitjà-Bobadilla and Alvarez-Pellitero 1993b and present study); but the distance between the cell membranes was similar to that found in desmosomes (Ham and Cormack 1979).

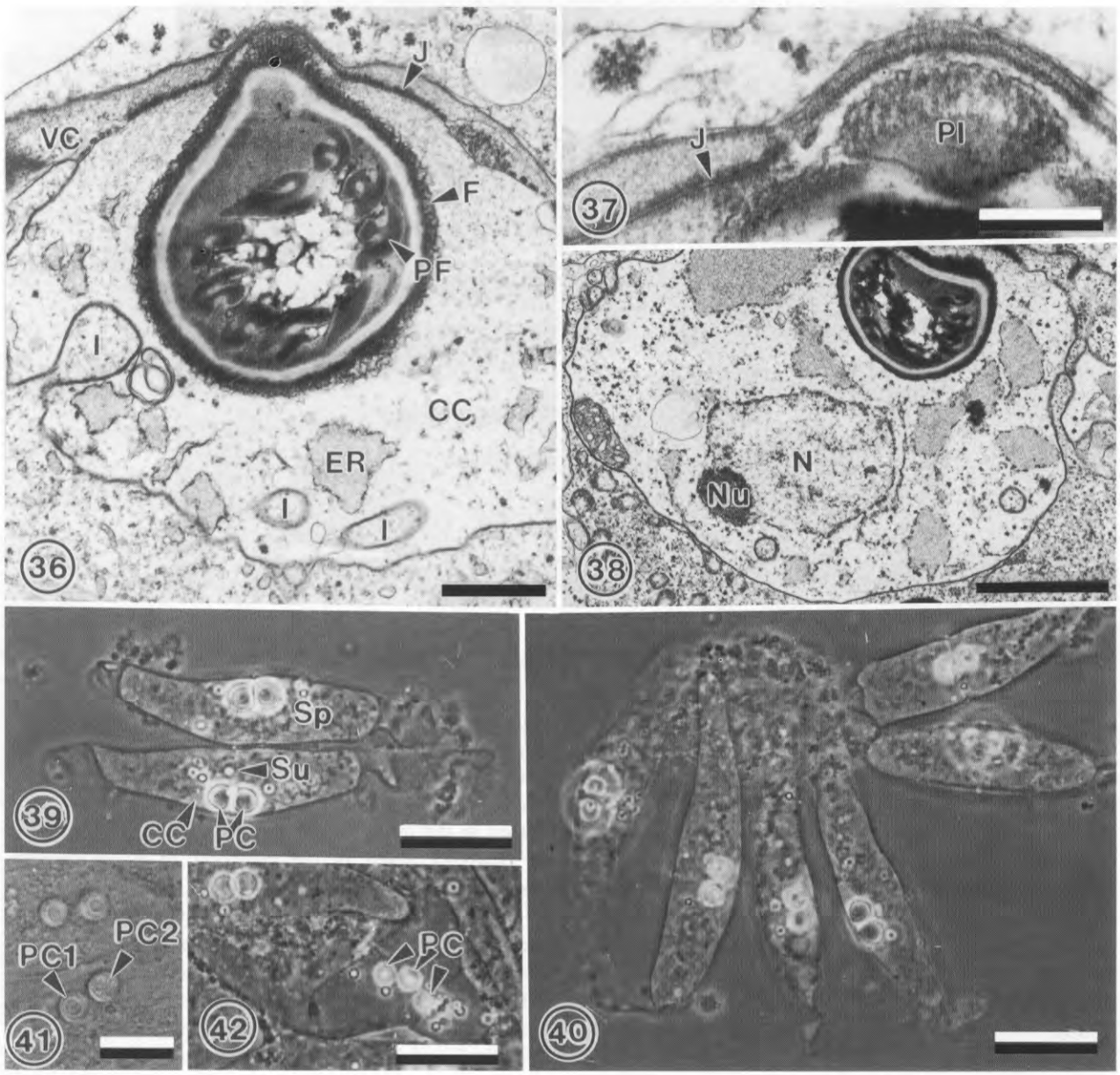


Fig. 36–40. *Ceratomyxa drepanopsettae*. TEM micrographs. **Fig. 36.** Polar capsule of maturing spore, surrounded by a fibrous coat (F). The polar filament (PF) is seen in cross-section. There are apical junctions (J) surrounded by fibrous material between the capsulogenic cell (CC) and the valvogenic cell (VC). There is some thickening of the outer membrane of the valvogenic cell. In the cytoplasm of the capsulogenic cell there are inflated cisternae of endoplasmic reticulum (ER), and invaginations (I) contain extensions of the sporoplasm. TEM. Bar = 1 μm. **Fig. 37.** Apical pore of polar capsule, closed by a plug (Pl), surrounded by a septate desmosome-like junction (J). TEM. Bar = 400 nm. **Fig. 38.** Nucleus (N) of capsulogenic cell, with small, peripheral nucleolus (Nu). TEM. Bar = 2 μm. **Fig. 39.** Free spores. Each spore contains two round polar capsules (PC) with coiled polar filaments. The capsulogenic cell (CC) has pale cytoplasm, contrasting with the granular cytoplasm of the sporoplasm (Sp), which extends to either side of the straight suture line (Su), one side being a little longer than the other. The valves are thin-walled, and have partially collapsed on either side of the sporoplasm. Squash preparation of fixed material. Phase contrast. Bar = 70 μm. **Fig. 40.** Group of spores attached at one end by the remains of the cytoplasm of the trophozoites. Squash preparation of fixed material. Phase contrast. Bar = 50 μm. **Fig. 41.** Polar capsules of free spores. One polar capsule is seen from the top or bottom, so that the spiral nature of the coil of the polar filament is visible (PC1); the other polar capsule is viewed from the side, so that seven turns of the coil can be seen (PC2). Squash preparation of fixed material. DIC. Bar = 70 μm. **Fig. 42.** Spore with three valves and polar capsules (PC). Squash preparation of fixed material. Phase contras. Bar = 40 μm.

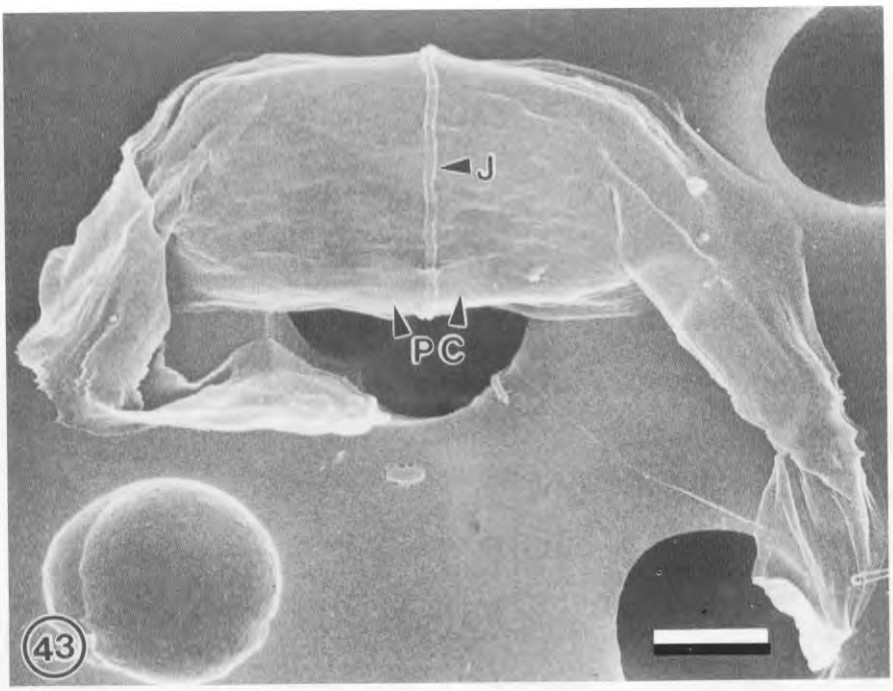


Fig. 43. *Ceratomyxa drepanopsettae*. Free spore on Millipore filter. The valves are thin-walled, attached by a junction (J). The faint outline of the polar capsules (PC) is visible. SEM. Bar = 5 μm.

Averintsev (1907, 1908a, 1909) found two vegetative nuclei in all trophozoites of *C. drepanopsettae*. The small, round trophozoites in which we saw only one nucleus were probably zygotes, since fusion of the two nuclei of the sporoplasm usually takes place before emergence (Shulman 1966). Some sectioned trophozoites had only one vegetative nucleus, but there might have been another nucleus not included in the section. Most myxosporean trophozoites have one vegetative nucleus but in some, including *C. drepanopsettae*, this nucleus divides to form two vegetative nuclei (Shulman 1966).

Averintsev (1908a, 1909) described an eccentric, dense aggregation of chromatin in the primary and secondary cell nuclei which he termed the "caryosome", and which we term a nucleolus. The nucleolus is commonly eccentric in myxosporeans (Shulman 1966), but the nucleoli of *C. drepanopsettae* seem to be unusual in that they are immediately adjacent to the nuclear membrane on one side, so that there are no nuclear pores in this region.

We found fewer small papillae on the trophozoites of *C. drepanopsettae* than in some other myxosporeans where they form a brush border, increasing the surface area of the trophozoite (Uspenskaya 1982). Pinocytosis is important in the utilization of food by all myxosporeans (Uspenskaya 1982), and was also observed at the surface of the trophozoites of another *Ceratomyxa* sp. (Diamant and Paperna 1989). Food vacuoles containing phagocytosed material have been described in trophozoites of several species of myxosporeans (Uspenskaya 1982). The feeding vacuoles we found did not contain identifiable cell remains, but appeared to be sites of active absorption and/or excretion. They occurred in the large, rounded region of the trophozoite, so probably aided in the transport of nutrients and excretions.

Mitochondria enlarged at the ends and constricted in the middle, as found in the trophozoites of *C. drepanopsettae*, were also described by Desportes and Théodoridès (1982) in *Leptotheca elongata*. Attachment of the trophozoites to the gallbladder wall by pseudopodia was described and illustrated by Averintsev (1908b). The pseudopodia did not appear to affect the gallbladder epithelial cells to the same extent as some myxosporeans such as *Myxobilatus mictospora*, which formed channels in the host cytoplasm (Booker and Current 1981). However, the dense layer at the surface of the pseudopodia of *C. drepanopsettae* appeared to be an attachment site to the microvilli, and some of the latter were irregular where trophozoites were present.

The first secondary cell in the trophozoite of *C. drepanopsettae* presumably forms by endogenous division, as in *Sphaerospora testicularis* (Sitjà-Bobadilla and Alvarez-Pellitero 1993b) but, as stated by these authors, the mechanism is unknown. Bundles of cytoplasmic microtubules are characteristic of myxosporean generative cells (Lom and Dyková 1992). The junctions found between some secondary cells presumably keep them together during sporoblast formation. We did not see

Table 2. Dimensions of spores of *Ceratomyxa drepanopsettae* (in μm).

	Auerbach (1912) (fixed)	Kudo (1918) (fresh)	Averintsev (1908a) (fixed)	Polyanskii (1955) (?)	Shulman (1966) (?)
Length of spore	ca.12–14	8–10	–	7.2–11.7	8–15.3
Thickness of sporoplasm	34	–	–	–	18–46
Total thickness of spore	ca. 56	64	50–80	45–92	45–92
Diameter of polar capsules	ca. 4–6	ca. 6	–	3.6–5.4	3.6–6

inter-cytoplasmic bridges between secondary cells as reported by Sitjà-Bobadilla and Alvarez-Pellitero (1993b).

Sporogenesis and structure of the free spores

Early sporoblast development in *C. drepanopsettae* is different from that in *C. shasta* (Yamamoto and Sanders 1979), where the sporoblast nuclei multiplied, apparently by fission, and then cell membranes were formed. Later development of some sporoblasts of *C. drepanopsettae* was, however, similar to that of *C. shasta*, where a group of two valvogenic, two capsulogenic and two sporoplasmic cells formed a sporoblast. Presumably the two sporoplasmic cells of *C. drepanopsettae* later fuse to form a binucleate sporoplasm, as in *C. shasta* (Yamamoto and Sanders 1979). The narrow part of the external tube was slightly narrower than that reported in *C. shasta* (Yamamoto and Sanders 1979). The wider portion may be equivalent to the dilated membrane-bound sac reported in *Sphaerospora angulata* (Desser et al. 1983), which became enlarged and spheroidal as it matured to form the polar capsule. The close connection between the capsulogenic and sporoplasmic cells, also reported by Averintsev (1908a, 1909), as well as the junctions between the pairs of valvogenic cells and sporoplasmic cells would probably help to keep the sporoblast together.

In some sporoblasts of *C. drepanopsettae* a small binucleate cell partially enveloped another cell before they differentiated, as in Figs. 21 and 28. Interpretation of the developmental sequence in sporoblasts where the secondary cells are not differentiated is very difficult, partly because we do not know the order in which the stages we observed occurred, and partly because it is difficult to interpret a three-dimensional structure from a two-dimensional section. In Fig. 21 the binucleate cell partially surrounds the other cell, but the other cell also extends small processes around the binucleate cell. The capsulogenic cell in Fig. 22 is also partially surrounded by the binucleate sporoplasm, but also has a process extending into the sporoplasm. The binucleate cells in Figs. 21 and 28 may therefore be sporoplasmic cells closely associated with capsulogenic cells. If this is the case, the sporoplasmic cells must have fused to form the binucleate sporoplasm earlier than in sporoblasts formed by the association of six secondary cells. The adjacent cells in Fig. 28 might be valvogenic cells, which would surround the binucleate sporoplasm and capsulogenic cells as described by Averintsev (1908a, 1909).

In other sporoblasts, one or two tertiary cells appeared within a large binucleate cell, either as a result of the binucleate cell surrounding another secondary cell, or as a result of endogenous division, as reported in *S. testicularis* by Sitjà-Bobadilla and Alvarez-Pellitero (1993b). These binucleate cells (Figs. 23, 24, and 28) are presumably valvogenic cells, since no other cells completely surround others in sporogenesis, although two nuclei have not, to our knowledge, been previously reported in a valvogenic cell. They all have microtubules in the peripheral cytoplasm, that appear to be associated with the formation of the long lateral processes of the valve of the spore. These microtubules were the same size as those described in other myxosporeans (Azevedo et al. 1989). The presence of two nucleoli in some tertiary cells indicates that they are dividing, presumably resulting in the formation of a capsulogenic and a sporoplasmic cell. The presence of sporoplasmosome-like bodies also suggests that some tertiary cells will become sporoplasmic cells.

Valvogenic cells had dense cytoplasm and nuclei like those of *Leptotheca elongata* (Desportes and Théodoridès 1982). Averintsev (1908a, 1909) found, as we did, that one sporoblast was more developed than the other in most trophozoites of *C. drepanopsettae*, and that some valvogenic cells became very large. Asynchrony is also common in disporous sphaerosporids (Sitjà-Bobadilla and Alvarez-Pellitero 1993b). The extensive glycogen stores and active pinocytosis at the surface of the valvogenic cells were presumably associated with the active growth and division of the enclosed tertiary cells.

Cell junctions between the valvogenic cells, and between these cells and the apical parts of the capsulogenic cells are characteristic of myxosporeans, and distinguish them from most protists (Azevedo et al. 1989). These junctions, unlike those we found between the trophozoites and between secondary cells, are usually associated with electron-dense material beneath

the cell membranes (Desportes and Théodoridès 1982, Azevedo et al. 1989 and Sitjà-Bobadilla and Alvarez-Pellitero 1993a,b). The extra support given by this material may be necessary in the mature spore but not the trophozoite, which is in a more protected environment.

The developing spore of *C. drepanopsettae* was similar to those of *C. globulifera* and *L. elongata* (Desportes and Théodoridès 1982) in that valvogenic processes gave rise to lateral extensions of the spore. Desportes and Théodoridès (1982) differentiated the spore of *L. elongata* from that of *C. globulifera* by the fact that there were extensions of the sporoplasm between the capsulogenic cells and the valvogenic cells in the former but not the latter. In *C. drepanopsettae* extensions of the sporoplasm surrounded all but the apical region of the capsulogenic cell, so the situation is intermediate between *L. elongata* and *C. globulifera*. The spore coat of developing spores of *C. drepanopsettae* was thinner than that observed in *C. shasta* (Yamamoto and Sanders 1979).

The polar capsules of *Ceratomyxa* species are usually rounded (Yamamoto and Sanders 1979), as described in *C. drepanopsettae*. The apical plug and external layer around the polar capsule of *C. drepanopsettae* were similar to those described by Desportes and Théodoridès (1982) in *L. elongata*. Extensions of cytoplasmic material from the sporoplasm into the capsulogenic cell were also described in *C. shasta* (Yamamoto and Sanders 1979).

A binucleate sporoplasm has been reported in many *Ceratomyxa* species (Sitjà-Bobadilla and Alvarez-Pellitero 1993a). Sporoplasmosomes are typically found in the sporoplasm of myxosporeans (Lom and Dyková 1992), and sporoplasmic cells could be recognised by the presence of sporoplasmosomes in the developing sporoblast of *C. shasta* (Yamamoto and Sanders 1979), as well as in our study. Elongate sporoplasmosomes were described by Sitjà-Bobadilla and Alvarez-Pellitero (1993a) in *C. labracis*; however, the sporoplasmosomes we found were circular and similar in size to those described by Azevedo et al. (1989). Since we found small dense bodies in smooth-membraned vesicles in the sporoplasm, the development of sporoplasmosomes may be similar to that of similar bodies in the primary cells of proliferative kidney disease in rainbow trout, *Oncorhynchus mykiss,* which seem to have their origin in Golgi complexes (Smith et al. 1984). Whorls of endoplasmic reticulum, like those we describe, were also found in the sporoplasm of *C. labracis* (Sitjà-Bobadilla and Alvarez-Pellitero 1993a).

Mature *C. drepanopsettae* spores were rarely found by Averintsev (1908a, 1909), Auerbach (1912), or Kudo (1918) and Averintsev (1908a, 1909) never found them free of the pseudoplasmodium. The general form of the thin-walled spore in Atlantic halibut was similar to that in Greenland halibut, *Reinhardtius hippoglossoides* (Wierzbicka 1988, 1990), except that we found a distinct, pale capsulogenic cell and bent or folded valves. Averintsev (1908a, 1909), however, drew the spores with a distinct capsulogenic cell and also found, as we did, some abnormal spores with 3 valves and 3 polar capsules. Spores with 3 valves were also described in *C. diplodae* and *C. labracis* (Sitjà-Bobadilla and Alvarez-Pellitero (1993a). Our measurements of the spores overlap with those obtained by other authors (Table 2). The clusters of developed spores that we described may have developed in trophozoites that were attached to each other.

Comparison between *Ceratomyxa drepanopsettae* and *C. ramosa*

Ceratomyxa ramosa was originally described by Averintsev (1907, 1908c), and has since been reported by Polyanskii (1955) and Zubchenko (1980). Although only drawings of *C. ramosa* are available, there seem to be distinctive features of the trophozoite that distinguish it from *C. drepanopsettae*. In *C. ramosa* two (less commonly one or three) processes extend from an expanded middle region containing the nuclei, and subdivide into several pseudopodia of different lengths which themselves subdivide into smaller branchlets that anastomose in some places and form reticulate structures. The endoplasm is finely granular and slightly vacuolated, and the ectoplasm is poorly developed (Averintsev 1908c, Shulman 1966). Although the trophozoites of *C. drepanopsettae* were variable, we seldom found trophozoites with nuclei in a central region; and while some trophozoites had complex, elongate pseudopodia, they also possessed an elongate process without pseudopodia, which contained prominent refractile granules instead of finely granular endoplasm. These trophozoites are therefore distinct from those of *C. ramosa*, and we agree with Polyanskii (1955) that, although the trophozoites are so variable and the spores so similar that *C. ramosa* may be a form of *C. drepanopsettae*, both species should be conserved, in case further studies confirm that *C. ramosa* is a separate species.

Myxidium sphaericum

The *M. sphaericum* trophozoites found in one Atlantic halibut were like those originally described by Thélohan (1895), in that many of the spherical trophozoites were found clustered together to form large masses, they were not larger than 20–22 μm in diameter, and had lobed pseudopodia. The spore size was similar to that described by Jayasri and Hoffman (1982).

Effects on host

There are many accounts of myxosporeans causing changes in the colour and consistency of the bile and the consistency of the gallbladder wall, and in some cases the bile duct was blocked (Noble 1957, Kabata 1967, Mitchell 1977, Lom 1984, Morrison et al. 1986, Lom and Dyková 1992, Alvarez-Pellitero and Sitjà-Bobadilla 1993). Although the bile in the Atlantic halibut we studied was cloudy because there were many parasites present, there seemed to be little effect on the gallbladder wall. Detached epithelial cells were associated with disintegrating parasites, so the damage may have been due to improper fixation of that region. However, accumulation of bile salts and cell debris in the heavily parasitized gallbladder indicates that the parasites may affect the physiology of the gallbladder, resulting in cell damage. It appears that *C. drepanopsettae* is only of concern if in sufficient numbers to interfere with the production or storage of bile, or to block the bile duct, resulting in loss of condition as reported by Dr. G. A. Bristow.

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