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Cryptosporidium molnari n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata* L. and *Dicentrarchus labrax* L.

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Abstract

Cryptosporidium molnari n. sp. is described from two teleost fish, the gilthead sea bream (*Sparus aurata* L.) and the European sea bass (*Dicentrarchus labrax* L.). The parasite was found mainly in the stomach epithelium and seldom in the intestine. Oocysts were almost spherical, with four naked sporozoites and a prominent residuum, and measured 3.23–5.45 × 3.02–5.04 (mean 4.72 × 4.47) μm in the type host, gilthead sea bream (shape index 1–1.17, mean 1.05). Sporulation was endogenous, as fully sporulated oocysts were found within the fish, both in the stomach epithelium and lumen, and in faeces. Oocysts and other stages of *C. molnari* fit most of the diagnostic features of the genus *Cryptosporidium*, but differ from hitherto described species, including piscine ones. All stages were located within a host contributed parasitophorous vacuole lined by a double host microvillar membrane. Merogonial and gamogonial stages appeared in the typical extracytoplasmic position, whereas oogonial and sporogonial stages were located deeply within the epithelium. Ultrastructural features, including the characteristic contact zone of the parasite with the host epithelial surface, were mostly coincident with those of other *Cryptosporidium* spp. Mitochondria were found in dividing meronts, merozoites, microgamonts and sporozoites. Pathological effects were more evident in gilthead sea bream, which also exhibited a clearly higher prevalence (24.4 versus 4.64% in sea bass). External clinical signs, consisting of whitish faeces, abdominal swelling and ascites, were rarely observed, in contrast with important histopathological damage. The wide zones of epithelium invaded by oogonial and sporogonial stages appeared necrotic, with abundant cell debris, and sloughing of epithelial cells, which detached to the lumen. No inflammation reaction was observed and the cellular reaction was limited to the cells involved in the engulfing of intraepithelial stages and debris, probably macrophages.

Keywords: *Cryptosporidium molnari* n. sp.; Coccidia; Ultrastructure; Histopathology; Teleostei; Aquaculture

1. Introduction

Cryptosporidium parasites are protozoan intracellular organisms that infect the gastrointestinal epithelial cells of a wide range of vertebrates, including humans. Among farmed animals, cryptosporidiosis is no longer considered an opportunistic disease, and the important economic losses caused by these coccidia, the zoonotic implications, and the difficulty to control them are of great concern (de Graaf et al., 1999). Much effort has been concentrated in the last decade to study the *Cryptosporidium parvum* group, the causative agent of human and animal cryptosporidiosis. However, important questions concerning many aspects of the parasite biology, pathology, immunology and epidemiology remain unanswered (Kosek et al., 2001). By comparison, the knowledge of piscine *Cryptosporidium* is still in its infancy, and little is known about the taxonomy, epidemiology, and pathology of isolates infecting fish. There have been several records of *Cryptosporidium* spp. in wild and cultured freshwater and marine hosts (Camus and López, 1996; Paperna and Vilenkin, 1996; Muench and White, 1997; see also the reviews of Fayer et al., 1997, 2000; Xiao et al., 2000). The interest in *Cryptosporidium* findings in such hosts is beyond taxonomic interest, as it is necessary to know if the species involved can be significant for human or domestic animal health. Cryptosporidiosis is a typical waterborne disease, and the survival of human species in sea water has been demonstrated, as well as the role of invertebrates as reservoirs (Tamburrini and Pozio, 1999; Fayer et al., 2000). However, the first trials to experimentally infect fish with *C. parvum* failed (Graczyk et al.,

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1996a), and nothing is known of the host range of the reported piscine species for fish or other animals, nor their impact under culture conditions.

In the Mediterranean area, with the increasing culture of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), new protozoan parasites have been described and problems derived from intensive culture have arisen. In this study we present morphological and ultrastructural data on a *Cryptosporidium* sp. which infects the gastric epithelial cells of both sea bass and sea bream. After detailed morphological studies at light and electron microscopy and comparison with known species, we describe it as a new species, named *Cryptosporidium molnari* n. sp.

2. Materials and methods

2.1. Fish

Several gilthead sea bream (*S. aurata*) and European sea bass (*D. labrax*) culture systems in the Mediterranean, Cantabric and Atlantic coasts of Spain were studied from 1998 to 2000, as part of a general survey on parasitic diseases. In this survey, 584 sea bream and 92 sea bass were examined in periodical samplings (group A), and 346 sea bream and 151 sea bass were sampled during outbreaks of mortalities or morbidity signs (group B). Fish were sacrificed after overexposure to the anaesthetic MS-222 (Sigma), complying with the current laws of Spain for animal use in experimental procedures. The fish were weighed, measured, and necropsied; their organs excised for fresh and histological examination for parasites.

2.2. Microscopic examination and histological procedure

Smears of stomach mucosal scrapings and faeces were examined fresh or after Giemsa staining. For histological studies, thin sections (1–3 μm) were obtained from material fixed in 10% buffered formalin and embedded in Technovit-7100 resin (Kulzer, Heraeus). They were stained with toluidine blue or with PAS. For TEM examination, small pieces of stomach were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), for 1 h at 4°C. Samples were washed several times with the same buffer, post-fixed in 1% (w/v) cacodylic OsO_4, dehydrated through a graded ethanol series, and embedded in Spurr's resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate. The sections were studied in Philips CM-10 and Hitachi H-800 TEM, operating at 75–100 kV. All ultrastructural observations were done from gilthead sea bream infected tissues, due to the scarcity of available material from sea bass.

For gilthead sea bream, photomicrographs of oocysts were digitally taken with a Spot camera (Diagnostic Instruments, Inc.), either from fresh material, or from histological sections. For sea bass, only histological images were obtained. Sporozoite digital images were taken from Giemsa-stained smears. All measurements were obtained from digital images with the software provided with the camera (Diagnostic Instruments, Inc.). Sporozoite width was taken from the wider part, at the level of the nucleus. All measurements are expressed in micrometers.

2.3. Statistical analysis

The lengths, widths, and shape index of oocysts taken from histological sections of sea bass and sea bream were compared using Student's *t*-test. Differences between sea bream oocysts measurements taken from fresh and histological images were also analysed with a Student's *t*-test. Values were considered significantly different if $P < 0.001$.

3. Results

3.1. Description of C. molnari n. sp.

3.1.1. Diagnosis

Oocysts, located deeply within the epithelium, spherical to subspherical, with a distinct colourless wall (Figs. 1–10). Longitudinal suture not visible in fresh preparations, but observed in histological sections (Fig. 8); oocyst residuum present, prominent, and PAS-positive, usually in a central position (Figs. 6 and 9). Four naked, curved, vermiform sporozoites in each oocyst (Figs. 7 and 8). The wider half of each sporozoite contained a nucleus. Excysted sporozoite measurements and sporulated oocysts measures are presented in Table 1. All the life stages were observed in the light microscopy studied material. Zygotes (Figs. 10 and 11), with numerous PAS-positive amylopectin granules

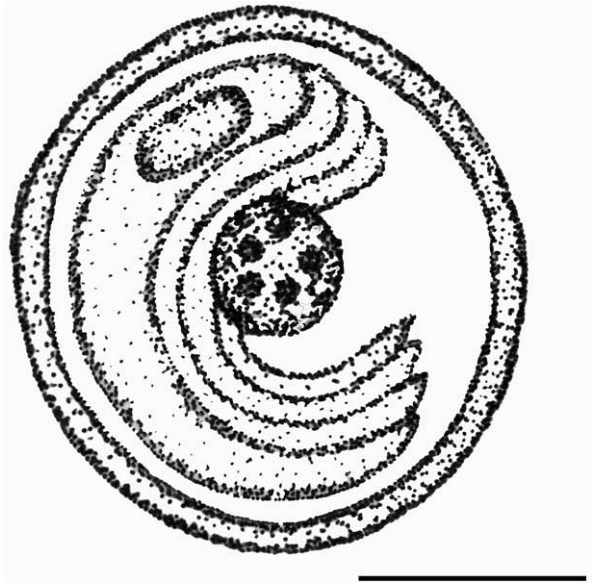
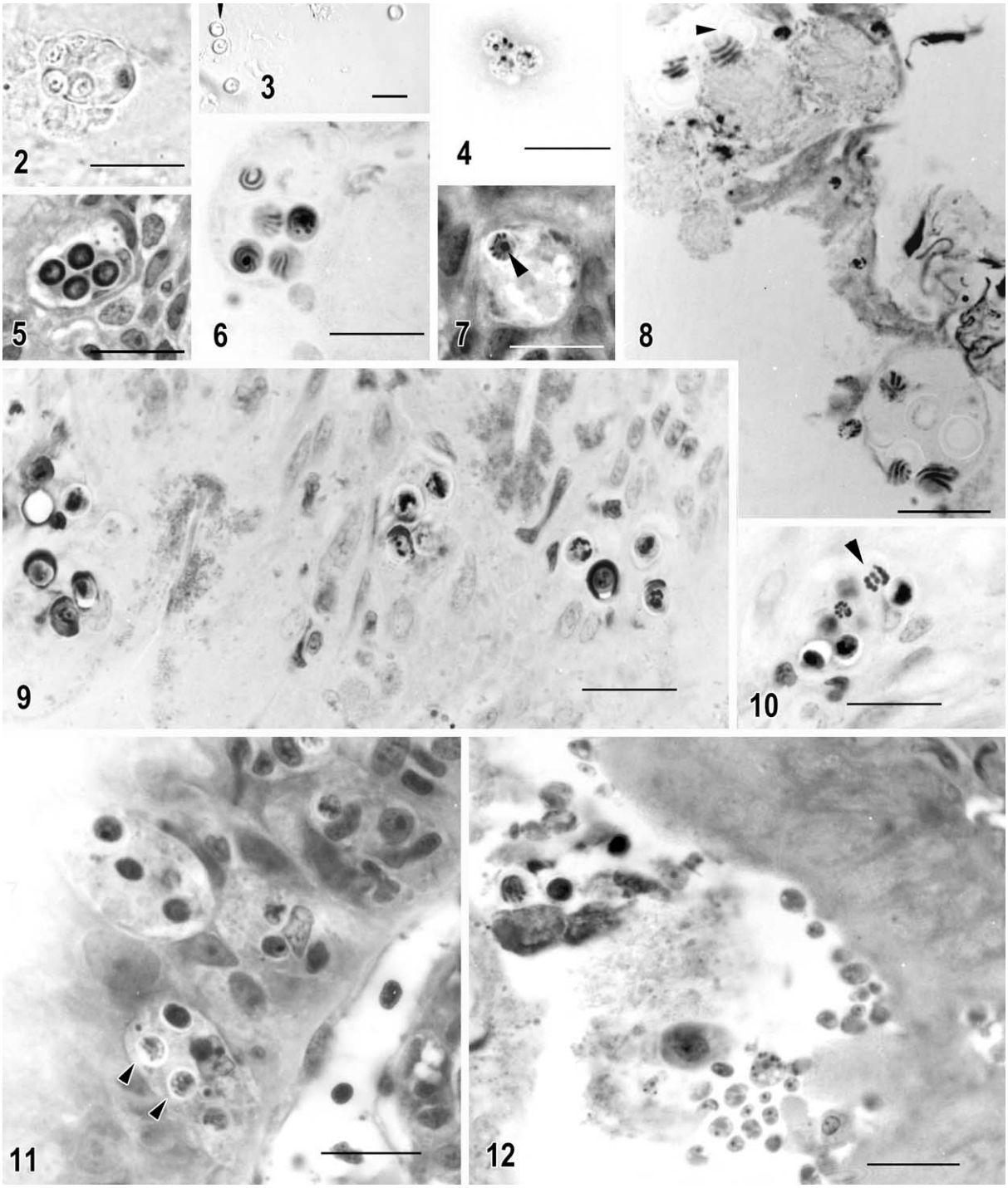


Fig. 1. Line drawing of *Cryptosporidium molnari* n. sp. oocyst. Bar, 2 μm.



Figs. 2–12. Photomicrographs of *Cryptosporidium molnari* from *Sparus aurata*. Figs. 2 and 3. Oocysts from fresh smears of stomach and faeces, respectively. Fig. 4. Giemsa stained non-sporulated oocysts from stomach. Notice the non-stained amylopectin granules. Figs. 5–12. Stomach semithin sections stained with toluidine blue (Figs. 5–8, 11, 12) or PAS (Figs. 9 and 10). Fig. 5. Group of oocysts in the stomach epithelium. Notice the host cell nucleus in a lateral position. Figs. 6–8. Sporulated oocysts. Notice the curved sporozoites, the oocyst residuum (arrowhead in Fig. 7), and the oocysts releasing sporozoites through the open suture (arrowhead in Fig. 8). Figs. 9 and 10. PAS-stained oocysts. Arrowhead in Fig. 10 points to the amylopectin granules. Fig. 11. Zygotes (arrowheads) located within the epithelium. Fig. 12. Meronts and early macrogamonts in extracytoplasmic position. Bars, 10 μm.

were also located deeply within the epithelium. Meronts of different sizes corresponding to different merogonial stages were seen lining the stomach epithelium. Trophozoites or initial meronts (Fig. 12) measured 1.8–5.7 × 1.2–4.6 μm, and divided meronts ranged 4.4–8.4 × 3.5–4.8 μm. Type I meronts with eight slender merozoites, as well as type II meronts with four merozoites were frequently observed, both in cross and longitudinal sections (Figs. 13–15). Micro-

Table 1
Measurements (in μm) of *Cryptosporidium molnari* in its hosts taken from different types of material[a]

Host	Type of material	Oocyst measurements							Sporozoite measurements		
		n	Length		Width		Shape index		*n*	Length	Width
Sparus aurata	Fresh	22	4.72 ± 0.53 (3.23–5.45)		4.47 ± 0.51 (3.02–5.04)	**	1.05 ± 0.05 (1.00–1.17)	**	–	–	–
	Histology	57	4.64 ± 0.29 (4.11–5.46)		4.13 ± 0.26 (3.63–4.85)		1.13 ± 0.07 (1.00–1.37)		–	–	–
	Smears		–		–		–		29	5.82 ± 0.72 (4.03–7.19)	0.99 ± 0.16 (0.83–1.47)
Dicentrarchus labrax	Histology	64	4.29 ± 0.22 (3.84–4.85)	*	3.99 ± 0.23 (3.52–4.64)	*	1.08 ± 0.05 (1.00–1.19)	*	–	–	–

[a] For each dimension the mean ± standard deviation is provided. The range appears in brackets. (–, data not determined). *indicates statistically significant differences between gilthead sea bream and sea bass oocysts measurements taken from histological material; **indicates statistically significant differences between measurements taken fresh and histological material from gilthead sea bream ($P < 0.001$).

gametocytes (Figs. 15 and 16) ranging 4.2–5.9 μm with numerous microgametes, and macrogametes (4.2–5.1 × 2.4–4.5 μm) were also detected.

3.1.2. Type host

Sparus aurata L. (gilthead sea bream), family Sparidae.

3.1.3. Other hosts

Dicentrarchus labrax L. (European sea bass), family Serranidae.

3.1.4. Prevalence of infection

Prevalence of infection was 6.5 and 25.43% in groups A and B of sea bream, and 11.95 and 4.64% in groups A and B of sea bass, respectively. Fingerling and juvenile were the main age classes infected.

3.1.5. Locality

The parasite was detected in cultured gilthead sea bream from fish farms located in different localities of Mediterranean, Cantabric and Atlantic Spanish waters, and from European sea bass from Mediterranean and Cantabric farms.

3.1.6. Location in host

Intracellular, preferentially in the epithelial cells of the stomach mucosa, and seldom in the intestinal epithelium. Merogonial and gamogonial stages were usually located in an extracytoplasmic position within the epithelial cell, whereas zygotes and oocysts were located mainly in the basal portion of the epithelium.

3.1.7. Sporulation

Endogenous. Fully sporulated oocysts were detected in the stomach or intestinal (rarely) epithelium and in the gastric or intestinal lumen, as well as in faeces.

3.1.8. Material deposited

Histological sections are deposited in the Museo Nacional de Ciencias Naturales, CSIC (Madrid, Spain): Colección Invertebrados, with holotype and paratype acquisition numbers MNCN 35.02/19 and MNCN 35.02/20, respectively.

3.1.9. Etymology

The species is named after the Hungarian parasitologist professor Kalman Molnár, for his contribution to the knowledge of piscine coccidia.

3.1.10. Statistical analysis

The statistical comparison of sea bream oocysts measurements taken from fresh and histological material showed that there were significant differences in the width and the shape index. Thus, fresh oocysts were significantly wider and more spherical than those observed in histological sections. In addition, the statistical analysis showed that sea bass oocysts were significantly more spherical and very slightly smaller than those from sea bream (Table 1).

3.2. Clinical signs and histopathology

Only gilthead sea bream with high intensity of infection exhibited clinical signs, consisting of whitish faeces, abdominal swelling and ascites. The histopathological study revealed the damage invoked by *C. molnari*. Meronts and gamonts in extracytoplasmic position apparently produced no harm to the fish tissue (Fig. 17). However, zygotes and oocysts produced a massive necrosis of epithelial cells. Single oocysts were rarely found and the initially infected cell appeared more or less affected, with the cytoplasm shrunk and the laterally located nucleus hypertrophied and eventually turned into pycnotic granules. More frequently, several oocysts appeared together, sometimes occupying wide zones of epithelium with a necrotic appearance, in which the remnants of the initially infected cells appeared strongly stained (Figs. 17–19). A vacuolar space surrounded some oocysts, which frequently appeared in groups engulfed by hypertrophied cells, probably macro-

phages. Sloughing of epithelial cells released oocysts into the stomach lumen in heavily infected fish, sometimes in groups (Figs. 7 and 12). In fish which had passed the infection, parasitic stages were already absent, but the stomach epithelial layer showed massive vacuolation and abundant debris appeared within the cells and in the lumen (Figs. 18 and 19). In spite of the destructive effect, no evident inflammatory host reaction was detected in infected stomachs, apart from the cells involved in the engulfing of groups of oocysts. Occasionally, rodlet cells were abundant close to infected areas (Fig. 19).

In sea bass no clear histopathological damage was observed as most of the infected fish had a low intensity of infection.

3.3. Ultrastructural observations

The ultrastructure of *C. molnari* n. sp. was studied exclusively from infected stomachs of gilthead sea bream. All stages were located within a host-contributed parasitophorous vacuole, initially formed at the microvillous surface of epithelial cells. Merogonial and gamogonial stages remained mainly in this extracytoplasmic position, whereas oogonial and sporogonial stages were only observed deeply in the epithelium.

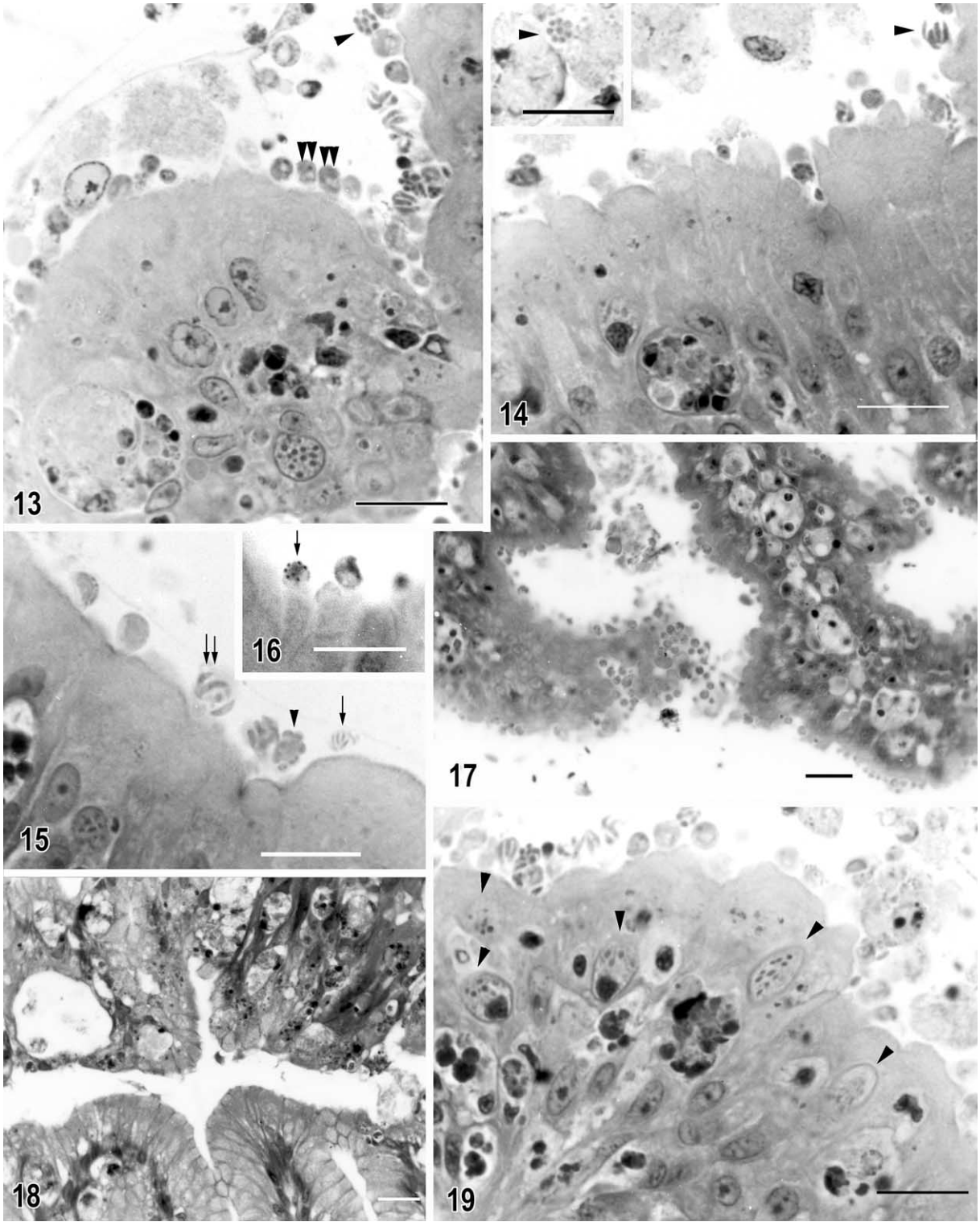
After contact of the sporozoite or merozoite with the epithelial cell, an electron-dense layer was formed at the host cell surface, which surrounded the parasite laterally (Figs. 20 and 21). The trophozoite became finally encircled by a double membrane of host origin corresponding to the villous border of the stomach mucosal epithelium. In the outermost membrane, short, widely spaced rudimentary microvilli were retained (Fig. 22). The electron density of the host cell at that contact point decreased later, but a web of microfilaments was appreciated (Fig. 23). The host and parasite membranes disappeared later, so the parasite became in contact with the cytoplasm of the epithelial cell. The parasite plasmalemma invaginated at the apex and formed abundant convolutions which increased its surface to form the so-called feeder organelle. Endocytic vesicles were seen close to it (Fig. 23). The parasite pellicle included an outer membrane and an inner more electron-dense membrane, especially evident at the contact point with the host cell (Figs. 21 and 24). Maturing trophozoites contained a single nucleus, numerous cytoplasmic ribosomes, and whorls of rough endoplasmic reticulum (rER). Small Golgi vesicles were seldom observed (Fig. 25). The nucleus, with scattered chromatin, underwent several divisions and trophozoites turned into meronts. In dividing meronts the cytoplasm retained the intense network of rER near to nuclei (Figs. 25 and 26). The electron-dense subpellicular layer was evident and acristate mitochondria were seldom observed (Fig. 26).

Meronts with fully developed merozoites were also detected (Figs. 27–30). Merozoites had an internal structure similar to that described for other coccidia, i.e. electron-dense granules, a double inner-membrane complex, small electron-dense inclusions, rophtries, micronemes, and many ribosomes (Figs. 27 and 30). Polar rings were scarcely visible at the apex, where the inner pellicular complex was discontinued (Fig. 30, inset). Subpellicular microtubules were scarcely visible (Fig. 30). Nuclei had scattered chromatin and only occasionally a nucleolus was detected. In some merozoites, a cisternal wall fringed by ribosomes encircled the nucleus (Fig. 28). Upon maturity, the meront's residuum, made of the feeder organelle and whorls of rER was conspicuous, and the fully formed merozoites separated from it. Eventually, the host cell membrane surrounding the meront lysed, and the merozoites become extracellular, able to infect other host cells. Meronts of types I (Fig. 27) and II (Fig. 30) were observed, the former being somewhat smaller and containing merozoites shorter and thinner than the latter. A mitochondrion was seen in some type II meronts (Fig. 30). Small electron-dense granules in the posterior part of merozoites of both types could be of carbohydrate nature (Figs. 29 and 30).

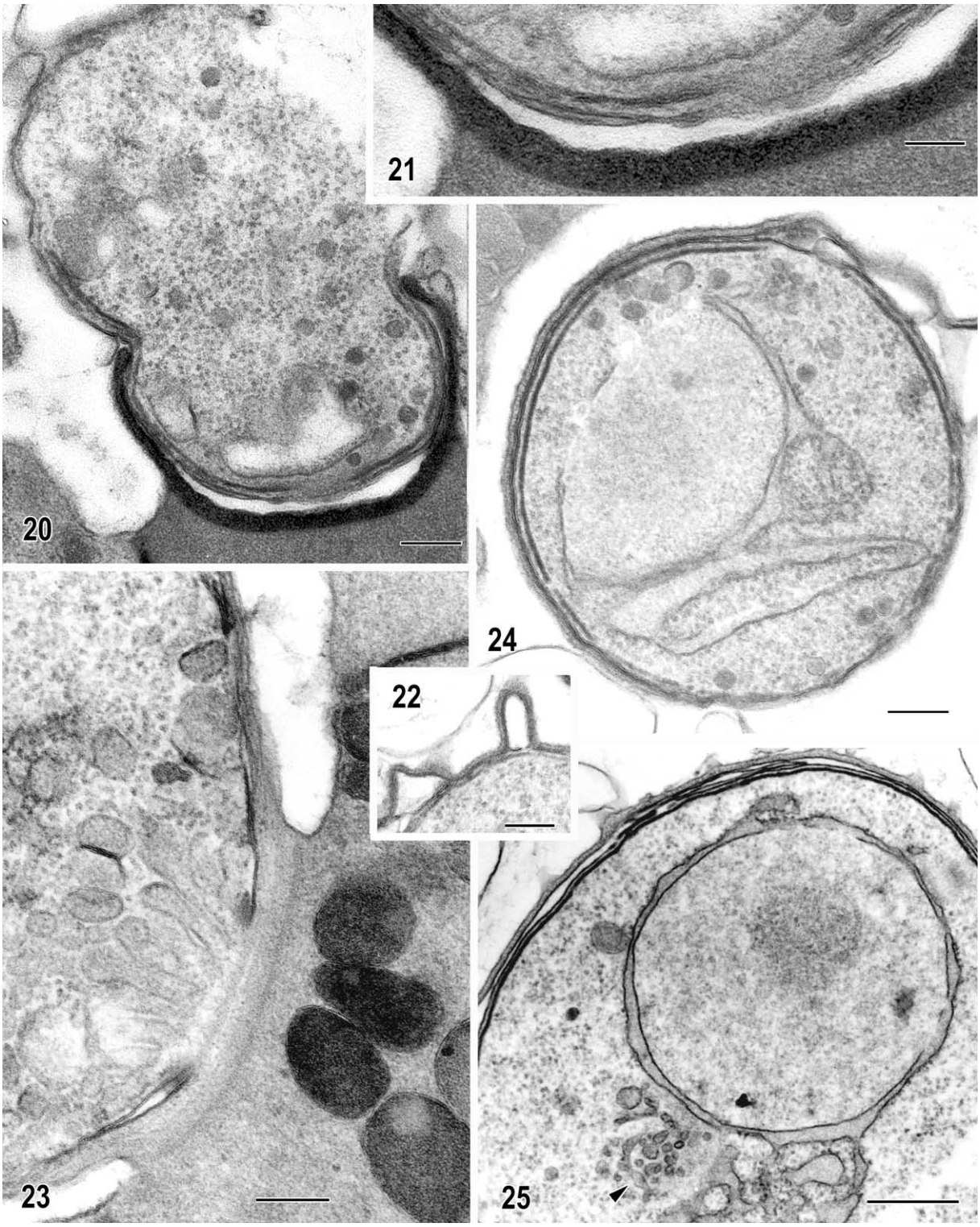
Microgamonts were relatively rare in comparison with other developmental stages, and also retained the microvilli on the outermost parasitophorous envelope (Figs. 31 and 32). Immature microgamonts contained small, numerous and compact nuclei arranged at the periphery (up to 12 nuclei were seen at the level of a section). The microgamont retained whorls of rER, abundant ribosomes, small Golgi vesicles and mitochondria (Fig. 31). Each microgamete separated from the gamont surface with a plasmalemma and an inner membrane, and lacked flagellum. Microtubules were seen in close proximity to the nuclear envelope (Fig. 32). No microgamete was seen in longitudinal section, thus its exact shape was not elucidated.

Early macrogamonts, also located in extracytoplasmic position, were indistinguishable from trophozoites until the polysaccharide granules formed. Those granules were identified as amylopectin granules. The cytoplasm was packed with ribosomes and contained extensive rER and lipidic vacuoles. Whorls of rER appeared sometimes in connection with the external nuclear membrane (Fig. 33). The extracytoplasmic macrogamonts retained the microvilli on the outermost parasitophorous envelop. Thus, the double membrane of the parasitophorous vacuole was seen beneath the macrogamont's plasmalemma (Figs. 33–35). In some places, the electron-dense inner membrane, as well as an additional trilayered membrane, probably involved in the wall formation, were evident (Figs. 34 and 35). At some time, and probably after fertilisation, the internalisation of the macrogamont or the zygote occurs, so zygotes and young or fully sporulated oocysts were found deeply in the epithelium within a vacuolar space. In the intraepithelial position, the microvillar surface was rarely retained (Fig. 36), as the parasitised cell was usually engulfed by another cell and soon destroyed (Fig. 41).

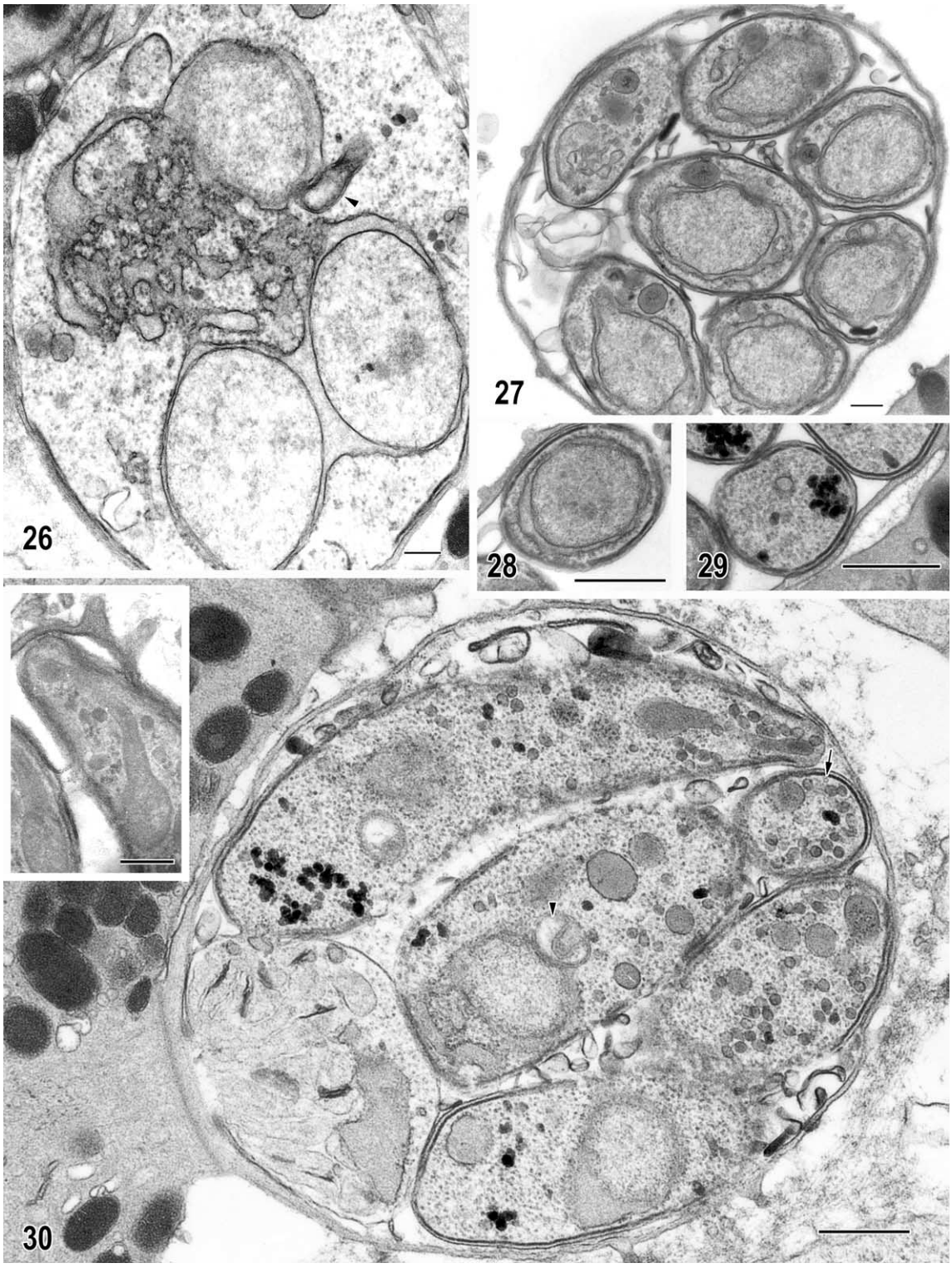
Zygotes and early oocysts contained many amylopectin granules and sometimes lipidic vacuoles. The suture was not



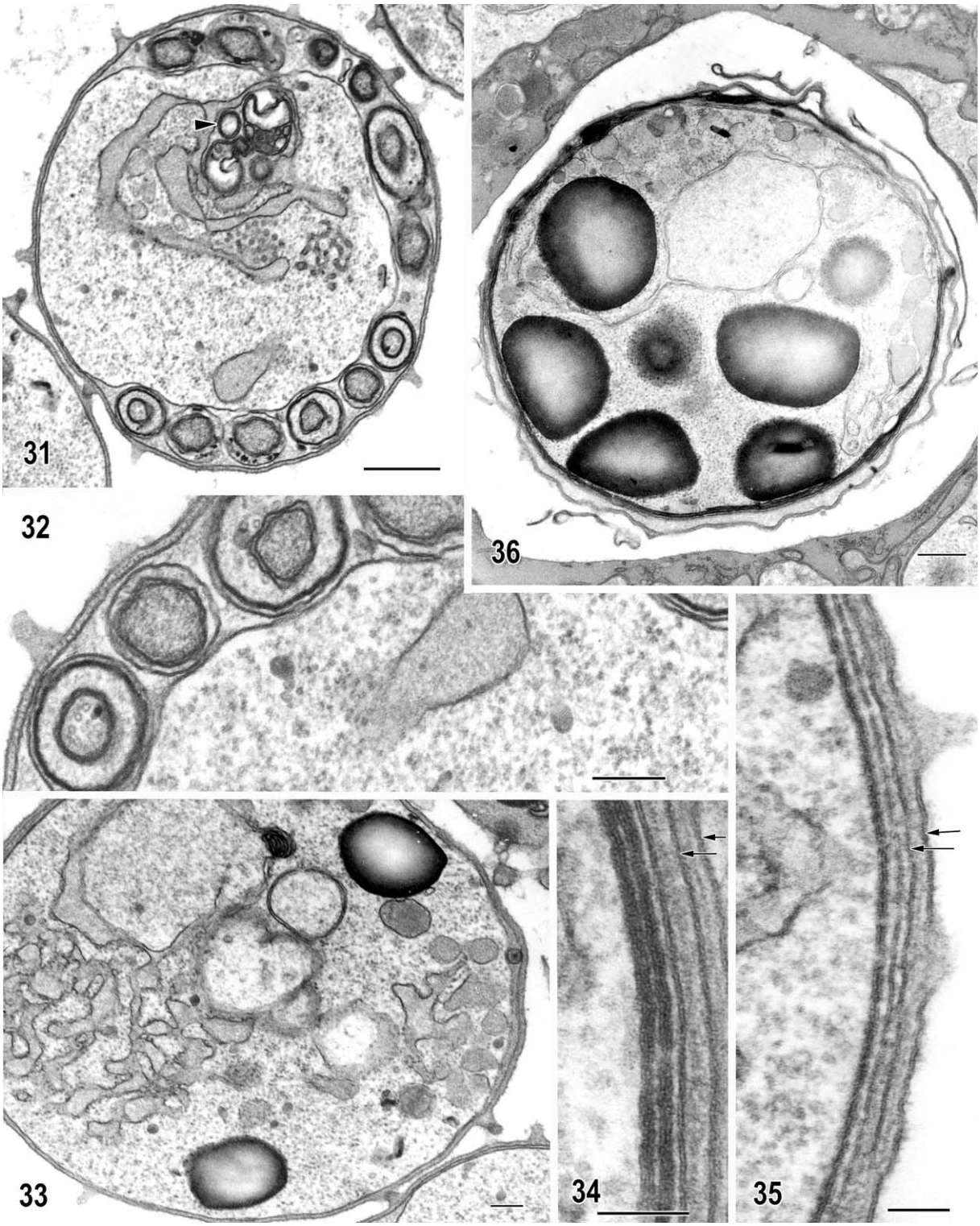
Figs. 13–19. Photomicrographs of *Cryptosporidium molnari* in toluidine blue-stained sections of *Sparus aurata* stomach. Figs. 13–16. Merogonial and gamogonial stages in extracytoplasmic position. Type-I meronts (arrowheads in Figs. 13–15), type-II meront (double arrow in Fig. 15), microgamonts in cross (arrow in Fig. 16) and longitudinal (arrow in Fig. 15) sections, and macrogamonts (double arrowhead in Fig. 13) can be seen. Fig. 17. Massive invasion by oocysts deep in the epithelium and other stages in extracytoplasmic position. Fig. 18. Vacuolation and necrosis of the epithelium in the late phases of the infection. Fig. 19. Cellular and parasitic debris in the epithelium together with abundant rodlet cells (arrowheads). Bars, 10 μm.



Figs. 20–25. Electron micrographs of merogonial stages of *Cryptosporidium molnari* from the stomach of *Sparus aurata*. Figs. 20 and 21. An early trophozoite in the process of being enveloped by the host cell membrane. Note the electron-dense band formed at the host cell, and the parasite pellicle. Fig. 22. Detail of a young meront showing the two microvillar membranes of the host cell covering the parasite. Fig. 23. Detail of a growing trophozoite at the host contact zone showing the invaginations of the parasite plasmalemma, endocytic vesicles, and a web of microfilaments in the host cell. Figs. 24 and 25. Young meronts starting the dividing process, with abundant ribosomes, whorls of rER and Golgi vesicles (arrowhead in Fig. 25). Bars, 0.1 μm in Fig. 21; 0.2 μm in Figs. 20, 23–25; and 0.5 μm in Fig. 25.

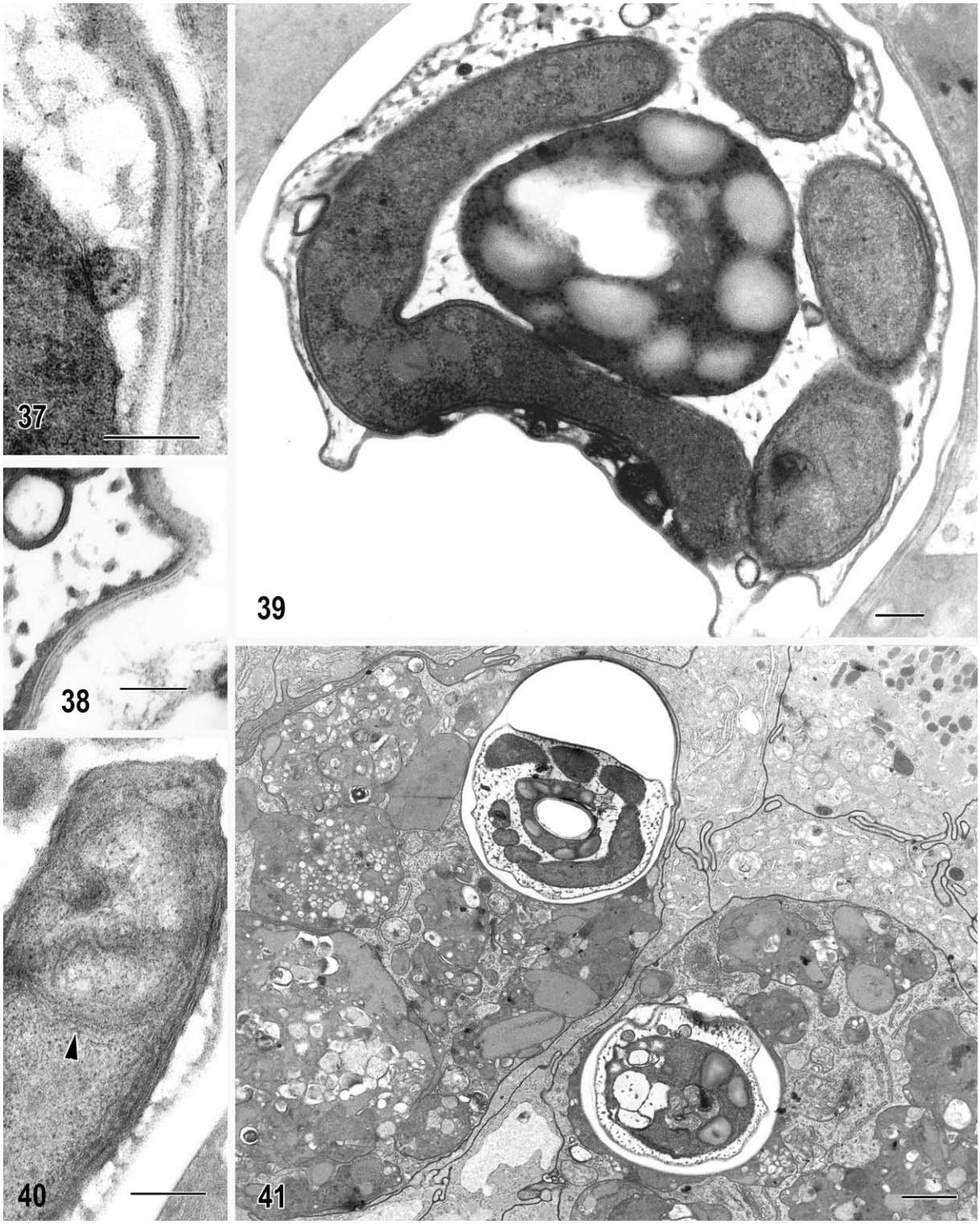


Figs. 26–30. Electron micrographs of merogonial stages of *Cryptosporidium molnari* from the stomach of *Sparus aurata*. Fig. 26. Dividing meront with nuclei in the process of karyocinesis. Notice the mitochondria (arrowhead) close to the whorls of rER. Figs. 27–29. Type-I meronts, with cross-sectioned merozoites containing rhopthries, the nucleus with a ring of ribosomes (Fig. 28), a bi-layered pellicle and small electron-dense granules (Fig. 29). Fig. 30. Oblique section of a type-II meront with merozoites still in contact with the feeding organelle. Merozoites have rophthries, micronemes, microtubules (arrow), a mitochondrion (arrowhead), small electron-dense granules, and preconoidal rings (inset). Bars, 0.2 μm in Figs. 26, 27, 30 inset; 0.5 μm in Figs. 28–30.



Figs. 31–36. Electron micrographs of gamogonial stages of *Cryptosporidium molnari* from the stomach of *Sparus aurata*. Fig. 31. Cross-section of a microgametocyte containing microgametes. In the microgamont remnant, mitochondria (arrowhead) and Golgi vesicles (arrow) can be seen. Notice the host microvilli in the outermost position. Fig. 32. Detail of microgametes in which microtubules are visible. Fig. 33. Macrogametocyte with amylopectin granules and rER. Figs. 34 and 35. Details of the membrane layers of macrogametocytes or zygotes. Arrows point to the two host-derived membranes, the remaining membranes are of parasite nature. Subpellicular membranes in Fig. 34 are probably involved in wall formation. Fig. 36. Young zygote located deeply in the epithelium in a vacuolar space. Amylopectin granules occupy most of the parasite and the host microvilli is still visible. Bars, 0.1 μm in Figs. 34 and 35; 0.2 μm in Figs. 32 and 33; and 0.5 μm in Figs. 31 and 36.

observed in the studied sections. Some membrane bounded inclusions resembling wall forming bodies type I appeared sometimes near the membrane (Fig. 36). About the time of sporulation the oocyst wall was formed. Both thick (Figs. 37 and 38) and thin (Fig. 39) walled oocysts were observed and the wall was frequently retracted inside the vacuole, mainly



Figs. 37–41. Electron micrographs of *Cryptosporidium molnari* oocysts from the stomach of *Sparus aurata*. Figs. 37 and 38. Details of thick walled oocysts showing outer and inner walls and the intermediate electron lucent layer. Fig. 39. Sporulated thin walled oocyst. One curved sporozoite in longitudinal section, three cross-sectioned sporozoites, and a large oocyst residuum can be seen. Fig. 40. Detail of a sporozoite with a mitochondrion (arrowhead). Fig. 41. Sporulated oocysts within a necrotic epithelial area, among cellular debris. Bars, 0.2 μm in Figs. 37–40; 1 μm in Fig. 41.

when the oocyst was engulfed by another cell in the inner part of the epithelium, together with the host cell remnants (Figs. 39 and 41). Thick walled oocysts had outer and inner walls separated by an electron-lucent layer (Figs. 37 and 38). The outer wall consisted of a thin layer sometimes with sparse filamentous material extending outwards. The inner layer had an inner particulate structure limited by a membrane.

Sporulated oocysts (Figs. 39 and 41) contained four naked sporozoites and a residual body filled with amylopectin granules, a lipid body and whorls of ER. Sporozoites had a pellicle formed by two bi-layered membranes, and were densely filled with ribosomes, micronemes and aggregates of small and large rounded bodies. A large mitochondrion was observed in some sporozoites (Fig. 40).

Oocysts were sometimes aggregated in zones occupied by degenerated host cells (Fig. 41). Some of them were apparently engulfed by a host cell (probably a macrophage) together with remnants of the initially infected cell, which appeared more electron-dense. In some cases infected cells underwent gradual degeneration and eventually disintegrated leaving aggregates of oocysts lying within intercellular spaces.

4. Discussion

4.1. Taxonomic remarks

Since the coccidian genus *Cryptosporidium* was first described by Tyzzer (1907) in the gastric glands of laboratory mouse, a large number of species has been reported in many other vertebrates, including humans. Levine (1984) reduced the number of valid species to four, one for each host class. According to Fayer et al. (1997), eight species should be recognised, but the latest tendency advocates for more valid species, in the light of more systematic morphological, biological and genetic characterisation of isolates from various hosts (Xiao et al., 2000; Fayer et al., 2000).

Cryptosporidium nasorum sp. from the tropical marine fish *Naso lituratus* (Hoover et al., 1981) is the only fish species included in the review of Fayer et al. (2000). Since then, other fish species have been reported to harbour *Cryptosporidium* or *Piscicryptosporidium* parasites, i.e. *Cyprinus carpio* (Pavlásek, 1983), *Plecostomus* spp. (Muench and White, 1997), *Lates calcarifer* (Glazebrook and Campbell, 1987), *Sciaenops ocellatus* (Camus and López, 1996), *Trichogaster leeri*, *Oreochromis* spp. and *S. aurata* (Paperna and Vilenkin, 1996), and *Scophthalmus maximus* (Alvarez-Pellitero et al., 1999). Other reports of cryptosporidiosis in teleosts were not confirmed with histology but involved identification of the organisms in faecal material, intestinal contents or scrapings (Rush et al., 1990; Gratzek, 1993; Stoskopf, 1993).

The currently described species showed most features of the genus *Cryptosporidium*, namely the extracytoplasmic development of merogonial and gamogonial stages, the absence of sporocysts, the absence of flagelles in microgametes, and the presence of four sporozoites. However, in our species, as in other piscine *Cryptosporidium* spp., sporulation takes place deeply within the epithelial cell, in contrast with species from other hosts. Paperna and Vilenkin (1996) created the genus *Piscicryptosporidium* to include some piscine species on the basis of such different oocyst location and the retention of residual microvilli. However, these apparently differential features have also been described in some mammalian *Cryptosporidium* spp. *Cryptosporidium parvum* has been found within some cells occasionally (Marcial and Madara, 1986; Beyer et al., 2000), and microvilli are usually retained in different mammalian species. Moreover, Morgan et al. (1999) pointed out the lack of biologic and genetic support for the genus *Piscicryptosporidium* (Morgan et al., 1999), and no *Cryptosporidium* sp.

Table 2
Oocyst measurements (in μm) of piscine *Cryptosporidium* spp.[a]

Species	Host	Site of infection	Oocyst measurements			Source
			n	Length*	Width	
C. nasoris	*Naso literatus*	Intestine	–	3.6	–	Hoover et al., 1981
Cryptosporidium sp.	*Cyprinus carpio*	Intestine		–	–	Pavlásek, 1983
Cryptosporidium sp.	*Lates calcarifer*	Intestine		–	–	Glazebrook and Campbell, 1987
Cryptosporidium sp.	*Sciaenops ocellatum*	Stomach	–	7	4	Camus and López, 1996
Cryptosporidium sp.	*Plecostomus* spp.	Stomach, intestine		–	–	Muench and White, 1997
Piscicryptosporidium reinchenbachklinkei	*Trichogaster leeri*	Stomach	–	2.4–3.18	2.4–3.0	Paperna and Vilenkin, 1996
Piscicryptosporidium cichlidis, previously *Cryptocystidium villithecum*	*Oreochromis* spp.	Stomach	–	4.0–4.70 (4.3 ± 0.46)	2.50–3.50 (3.25 ± 0.4)	Paperna and Vilenkin, 1996
Piscicryptosporidium sp., previously *Chloromyxum-like*	*Sparus auratus*	Stomach	32	3.1 ± 0.5	–	Paperna and Vilenkin, 1996

[a] *Corresponds to the diameter when only one measurement is given. –, not available in the literature.

from fish has been genotyped yet. Further molecular studies on them, as well as on many other species, would help to elucidate their definitive taxonomic position and the organisation of this controversial group, including the revision of the diagnostic features of the genus. Therefore, with the present state of knowledge we ascribe our species to the genus *Cryptosporidium*.

We regard the species found in sea bass and sea bream as unique one and consider that the statistical difference found in oocyst measurements of both hosts is not biologically significant, and could be due to intraspecific variation of oocyst size, as described in other *Cryptosporidium* spp. (Chacín-Bonilla, 1995; Sréter et al., 2000). This fact has been attributed to the influence of host conditions. Moreover, we have recently transmitted the cryptosporidial infection from gilthead sea bream to sea bass, both by oral inoculation and by cohabitation (unpublished data).

The described species differs from all the known species in the genus. Regarding all the mammalian and avian species, our species has a different oocyst location in the epithelium, but the oocysts measurements are within the oocyst range for *C. parvum*. Obviously, only a genetic study will support our hypothesis. Nevertheless, preliminary immunohistochemistry tests with monoclonal antibodies against *C. parvum* have been negative (Dr L. Ortega, personal communication). This is quite relevant, considering that commercial antibody tests produce cross-reaction with a variety of *Cryptosporidium* species (Graczyk et al., 1996b). In addition, the finding of our species was not casual, but was detected after examining hundreds of animals after different culture conditions from different regions (Sitjà-Bobadilla and Alvarez-Pellitero, 2001).

The comparison with other piscine *Cryptosporidium* is difficult, as some descriptions are incomplete, are based on data from very few fish or few oocysts, or measurements are not even reported (Table 2). *Cryptosporidium nasoris*, *Piscicryptosporidium reinchenbachklinkei* and *Piscicryptosporidium cichlidis* oocysts are smaller than those of *C. molnari*, and the species found in *Sciaenops ocellatum* has larger oocysts. Also, given the inaccuracies of measuring such small oocysts, and the shrinkage produced by some histological procedures, the species reported previously in the gilthead sea bream (Paperna and Vilenkin, 1996), is probably the same as the one found by us. Thus, with the present state of knowledge, and on the basis of the presented data, our species must be considered a new one.

4.2. Ultrastructural aspects

Data on *Cryptosporidium* sp. ultrastructure are scarcer than those dealing with other Apicomplexa genera, as *Eimeria*, *Toxoplasma* or *Sarcocystis* (Chobotar and Scholtyseck, 1982). However, some ultrastructural features are known for several *Cryptosporidium* spp., i.e. the mammalian species *C. parvum* (Bird and Smith, 1980; Current, 1989; Fayer et al., 1997), *Cryptosporidium felis* (Iseki, 1979), *Cryptosporidium wrairi* (Vetterling et al., 1971) and *Cryptosporidium muris* (Uni et al., 1987); the avian *Cryptosporidium baileyi* (Cheadle et al., 1999) and *Cryptosporidium anserinum* (Proctor and Kemp, 1974); the reptilian *Cryptosporidium saurophilum* (Koudela and Modrý, 1998), and the piscine species (described in the genus *Piscicryptosporidium*) from the gourami and from cichlid fish (Landsberg and Paperna, 1986; Paperna and Vilenkin, 1996).

The ultrastructural study of *C. molnari* has demonstrated the parallelism of its main features with those of other *Cryptosporidium* spp. The invasion of the host cells also occurs in a similar way to other species, i.e. through contact with epithelial surface and lateral growth of host cell membranes to form a parasitophorous vacuole (Vetterling et al., 1971; Marcial and Madara, 1986; Fayer et al., 1997, 2000). The folding of the contact zone to form the 'feeding organelle' was also observed. The presence of microfilaments at the contact zone also resembles that described in other *Crysptosporidium* spp. (Fayer et al., 1997), though Beyer et al. (2000) describe tubular-like structures in *C. parvum*, and Uni et al. (1987) indicated an indented border separating the projection of the parasite from the filamentous process of the host cell in *C. muris*. The subpellicular membrane corresponding to the electron-dense collar (Uni et al., 1987; Fayer et al., 1997), was also evident, especially at the contact between parasite and host cell membranes.

The extracytoplasmic stages of *C. molnari*, i.e. meronts (types I and II), merozoites and gamonts, share most of the features of the *Cryptosporidium* spp. studied by TEM. Some elements of the apical complex were scarcely distinguishable in merozoites of *C. molnari*. Fayer et al. (1997) also indicated the lack of typical polar rings in *Cryptosporidium* spp., in contrast with Uni et al. (1987) who described three preconoidal rings and a conoid in *C. muris*.

According to Chobotar and Scholtyseck (1982), a pellicle with three unit membranes is characteristic of several coccidia, including the plasmalemma and the inner pellicle complex. Uni et al. (1987) described three unit membranes in the pellicle of *C. muris* merozoites. An outer membrane and a subpellicular complex formed by two membranes was also found in *C. molnari*, though the outer membrane appeared sometimes as a bi-layered one. The trilamellar pellicle may persist or not throughout the gametogenesis, as described in other coccidia (Chobotar and Scholtyseck, 1982). We observed subpellicular microtubules similar to those described by Uni et al. (1987) in *C. muris*.

Macrogamonts and microgamonts also had the typical structure. Inclusions considered as whorl forming bodies were rarely observed in macrogamonts and were similar to whorl forming bodies 1 (Chobotar and Scholtyseck, 1982). Thus, we were not able to confirm the presence of both types of WFB in thick walled oocysts and their

absence in thin walled oocysts as described by Fayer et al. (1997).

The location of sporulating or sporulated oocysts of *C. molnari* more or less deeply in the epithelium and not in extracytoplasmic position, was confirmed by the TEM study. Thin and thick walled oocysts, as described for most *Cryptosporidium* spp., also seem to exist. Though the steps in the wall formation were not ascertained, three main layers appeared in the wall as in *C. parvum* (Fayer et al., 1997; Harris and Petry, 1999). The secondary loss of the external layer of the thick walled oocyst, as in *C. parvum* (Current, 1989), could also occur. In the case of oocysts from fish, Landsberg and Paperna (1986) described oocysts having a double-layered thick wall in *Cryptosporidium* sp., and Paperna and Vilenkin (1996) reported a firm wall in *P. reichenbachklinkei*, but the electron micrographs did not show the wall in detail.

We ascertained the presence of apparently acristate mitochondria in several stages of *C. molnari*. The presence of mitochondria in *Cryptosporidium* spp. has been controversial for many years. Although ultrastructural (Tetley et al., 1998), and biochemical and molecular (Denton et al., 1996) studies suggested its absence in *C. parvum*, mitochondria had been mentioned by Moriya (1989) in macrogametes, young oocysts and sporozoites, and they were recently demonstrated in sporozoites (also confirmed by genetic studies, Riordan et al., 1999) and merozoites (Beyer et al., 2000). These organelles have also been reported in the microgamete of *Cryptosporidium* sp. from mice (Göbel and Brändler, 1982), and in merozoites and macrogametes of *C. muris* (Uni et al., 1987). The plastid, other organelle present in different Apicomplexa (McFadden et al., 1997), was not demonstrated in our material, nor it has been confirmed in other *Cryptosporidium* spp. The plastid-like organelle mentioned by Tetley et al. (1998) in *C. parvum*, is probably a mitochondrion according to Riordan et al. (1999).

4.3. Pathological effects

Clinical signs of gastrointestinal cryptosporidosis in avian and mammalian hosts include diarrhoea, lethargy, apathy and depression, anorexia, lower pigmentation, growth retardation, and mortality (de Graaf et al., 1999; Sréter and Varga, 2000). In some infected fish, anorexia, regurgitation of food, emaciation, atrophy of skeletal muscle, and tucked abdomen have been reported (Hoover et al., 1981; Gratzek, 1993; Camus and López, 1996), whereas in other cases, no pathological or clinical signs have been detected (Pavlásek, 1983; Landsberg and Paperna, 1996). In gilthead sea bream, only when intensity of infection was high, abdominal swelling and ascites accumulation appeared. Also, mortalities associated to *C. molnari* have been reported in some fingerling stocks (Sitjà-Bobadilla and Alvarez-Pellitero, 2001). The lack of signs in infected sea bass was probably due to the low intensity of infection registered in most of the infected fish. Similarly, asymptomatic infections are commonly reported in some rodent species (Vetterling et al., 1971) and piglets (de Graaf et al., 1999).

As evidenced by our histological study, the accumulation of *C. molnari* oocysts within the mucosal tissue, and the consecutive necrosis, vacuolation, and sloughing of epithelial cells are clear signs of the pathological impact of this parasite on the fish. This is also the case of other piscine species, in which the parasite distorted the normal mucosal architecture (Landsberg and Paperna, 1986; Camus and López, 1996). In non-piscine hosts, where the entire cycle is extracytoplasmic, *Cryptosporidium* is regarded as a minimally invasive mucosal pathogen, and yet histopathological findings are obvious (de Graaf et al., 1999; Sréter and Varga, 2000).

Concerning the host cell reaction, in the present work no cellular reaction was detected in infected stomachs. In barramundi, a *Cryptosporidium* sp. was found in association with inflammatory cells in the intestinal lamina propria (Glazebrook and Campbell, 1987). Also, red drum (*S. ocellatus*) with gastric cryptosporidiosis presented submucosa and lamina propria infiltrated with eosinophilic granule cells, lymphocytes and macrophages (Camus and López, 1996). This is also the case in other non-piscine hosts, as lizards (Koudela and Modrý, 1998) and mammals (Laurent et al., 1999), in which an inflammatory leucocytic infiltrate can be observed in the underlying lamina propria. The involvement of macrophages in response to *C. parvum* has also been ultrastructurally demonstrated (Marcial and Madara, 1986; Beyer et al., 2000). It is also probable that macrophages or other phagocytic cells are involved in the engulfing of *C. molnari* stages when they appear occupying large zones associated with cellular debris.

In the *Cryptosporidium* found in the stomach of cichlid fish, oocysts were very rarely released to the lumen, contrary to their frequent luminal location in our sea bream. As happens in mammals (O'Donoghue, 1995), the sporulated oocysts released in the lumen could excyst in the same animal and contribute to autoinfection and persistent infections.

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References

Alvarez-Pellitero, A., Redondo, M.J., Sitjà-Bobadilla, A., Macías, A., Riaza, A., Padrós, A., 1999. Epidemiological study of an intestinal coccidiosis of cultured turbot (*S. maximus* L.). Fifth International Symposium on Fish Parasites. Book of Abstracts. Ceske Budejovice, Czech Republic, pp. 4.

Beyer, T.V., Svezhova, N.V., Sidorenko, N.V., Khokhlov, S.E., 2000. *Cryptosporidium parvum* (Coccidia, Apicomplexa): some new ultrastructural observations on its endogenous development. Eur. J. Protistol. 36, 151–9.

Bird, R.G., Smith, M.D., 1980. Cryptosoridiosis in man: parasite life cycle and fine structural pathology. J. Pathol. 132, 217–33.

Camus, A.C., López, M.K., 1996. Gastric cryptosporidiosis in juvenile red drum. J. Aquat. Anim. Health 8, 167–72.

Chacín-Bonilla, L., 1995. Cryptosporidiosis en humanos. Invest. Clin. 36, 207–50.

Cheadle, M.A., Toiviokinnucan, M., Blagburn, B.L., 1999. The ultrastructure of gametogenesis of *Cryptosporidium baileyi* (Eimeriorina; Cryptosporiidae) in the respiratory tract of Broiler chickens (*Gallus domesticus*). J. Parasitol. 85, 609–15.

Chobotar, B., Scholtyseck, E., 1982. Ultrastructure. In: Long, P.L. (Ed.). The Biology of the Coccidia, University Park Press, Baltimore, MD, pp. 101–65.

Current, W.L., 1989. *Cryptosporidium* spp. In: Walzer, P.D., Genta, R.M. (Eds.). Parasitic Infections in the Compromised Host, Marcel Dekker, New York, NY, pp. 281–341.

Denton, H., Brown, S.M.A., Roberts, S.M.A., Alexander, J., McDonald, V., Thong, K.-W., Coombs, G.H., 1996. Comparison of the phosphofructokinase and piruvate kinase activities of *Cryptosporidium parvum*, *Eimeria tenella* and *Toxoplasma gondii*. Mol. Biochem. Parasitol. 76, 23–29.

Fayer, R., Speer, C.A., Dubey, J.P., 1997. The general biology of *Cryptosporidium*. In: Fayer, R. (Ed.). *Cryptosporidium* and Cryptosporidiosis, CRC Press, Boca Raton, FL, pp. 1–41.

Fayer, R., Morgan, U., Upton, S.J., 2000. Epidemiology of *Cryptosporidium*: transmission, detection and identification. Int. J. Parasitol. 30, 1305–22.

Glazebrook, J.C., Campbell, R.S.F., 1987. Diseases of Barramundi (*Lates calcarifer*) in Australia: a review. In: Copland, J.W., Grey, D.L. (Eds.). Management of Wild and Cultured Sea Bass/Barramundi (*Lates calcarifer*), Australian Centre for International Agricultural Research, Canberra, pp. 204–6.

Göbel, E., Brändler, U., 1982. Ultrastructure of microgametogenesis, microgametes and gametogony of *Cryptosporidium* sp. in the small intestine of mice. Protistologica 18, 331–44.

de Graaf, D.C., Vanopdenbosch, E., Ortega-Mora, L.M., Abbassi, H., Peeters, J.E., 1999. A review of the importance of cryptosporidiosis in farm animals. Int. J. Parasitol. 29, 1269–87.

Graczyk, T.K., Fayer, R., Cranfield, M.R., 1996a. *Cryptosporidium parvum* is not transmissible to fish, amphibians, orreptiles. J. Parasitol. 82, 748–51.

Graczyk, T.K., Cranfield, M.R., Fayer, R., 1996b. Evaluation of commercial enzyme immunoassay (EIA) and immunofluorescent antibody (IFA) test kits for detection of *Cryptosporidium* oocysts of species other than *Cryptosporidium parvum*. Am. J. Trop. Med. Hyg. 54, 274–9.

Gratzek, J.B., 1993. Parasites associated with fresh-water tropical fishes. In: Stoskopf, M.E. (Ed.). Fish Medicine, Saunders, Philadelphia, PA, pp. 573–90.

Harris, J.R., Petry, F., 1999. *Cryptosporidium parvum*: structural components of the oocyst wall. J. Parasitol. 85, 839–49.

Hoover, M.D., Hoerr, F.J., Carlton, W.W., Hinsman, E.J., Ferguson, H.W., 1981. Enteric cryptosporidiosis in a naso tang, *Naso lituratus* Bloch and Scheiner. J. Fish Dis. 4, 425–8.

Iseki, M., 1979. *Cryptosporidium felis* sp. n. (Protozoa: Eimeriorina) from the domestic cat. Jpn. J. Parasitol. 28, 285–307.

Kosek, M., Alcantara, C., Lima, A.A.M., Guerrant, R.L., 2001. Cryptosporidiosis: an update. Lancet Infect. Dis. 1, 262–9.

Koudela, B., Modrý, D., 1998. New species of *Cryptosporidium* (Apicomplexa: Cryptosporidiidae) from lizards. Folia Parasitol. 45, 93–100.

Landsberg, J.H., Paperna, I., 1986. Ultrastructural study of the coccidian *Cryptosporidium* sp. from stomachs of juvenile cichlid fish. Dis. Aquat. Org. 2, 13–20.

Laurent, F., McCole, D., Eckmann, L., Kagnoff, M.F., 1999. Pathogenesis of *Cryptosporidium parvum* infection. Microbes Infect. 2, 141–8.

Levine, N.D., 1984. Taxonomy and review of the coccidian genus *Cryptosporidium* (Protozoa, Apicomplexa). J. Protozool. 31, 94–98.

Marcial, M.A., Madara, J.L., 1986. *Cryptosporidium*: cellular localization, structural analysis of absorptive cell parasite membrane-membrane interactions in guinea pigs, and suggestion of protozoan transport by M cells. Gastroenterology 90, 583–94.

McFadden, G.I., Reith, M., Munholland, J., Lang-Unnasch, N., 1997. Plastids in apicomplexan parasites. Pl. Syst. Evol. 11S, 261–78.

Morgan, U.M., Xiao, L., Fayer, R., Lal, A.A., Thompson, R.C.A., 1999. Variation in *Cryptosporidium*: towards a taxonomic revision of the genus. Int. J. Parasitol. 29, 1733–51.

Moriya, K., 1989. Ultrastructural observations on oocysts, sporozoites and oocyst residuum of *Cryptosporidium muris* (Strain RN 66). J. Osaka City Med. Cent. 38, 177–201.

Muench, T.R., White, M.R., 1997. Cryptosporidiosis in a tropical freshwater catfish (*Plecostomus* spp.). J. Vet. Diagn. Invest. 9, 87–90.

O'Donoghue, P.J., 1995. *Cryptosporidium* and cryptosporidiosis in man and animals. Int. J. Parasitol. 25, 139–95.

Paperna, I., Vilenkin, M., 1996. Cryptosporidiosis in the gourami *Trichogaster leeri*: description of a new species and a proposal for a new genus, *Piscicryptosporidium*, for species infecting fish. Dis. Aquat. Org. 27, 95–101.

Pavlásek, I., 1983. *Cryptosporidium* sp. in *Cyprinus carpio* Linné, 1758 in Czechoslovakia. Folia Parasitol. 30, 248.

Proctor, S.J., Kemp, R.L., 1974. *Cryptosporidium anserinum* sp. n. (Sporozoa) in a domestic goose *Anser anser* L., from Iowa. J. Protozool. 21, 664–6.

Riordan, C.E., Langreth, S.E., Sanchez, L.B., Kayser, O., Keithly, J.S., 1999. Preliminary evidence for a mitochondrion in *Cryptosporidium parvum*: phylogenetic and therapeutic implications. J. Eukaryot. Microbiol. 46, 52S–5S.

Rush, B.A., Chapman, P.A., Ineson, R.W., 1990. A probable waterborne outbreak of cryptosporidiosis in the Sheffield area. J. Med. Microbiol. 32, 239–42.

Sitjà-Bobadilla, A., Alvarez-Pellitero, P., 2001. *Cryptosporidium* sp. (Apicomplexa: Cryptosporidiidae), an ubiquitous parasite in gilthead sea bream (*Sparus aurata*): epidemiological survey in Spanish cultures. Tenth International Conference of the EAFP on Diseases of Fish and Shellfish. Book of Abstracts. Dublin, Ireland, pp. O-52.

Sréter, T., Varga, I., 2000. Cryptosporidiosis in birds – a review. Vet. Parasitol. 87, 261–79.

Sréter, T., Kovács, G., Da Silva, A.J., Pieniazek, N.J., Széll, Z., Dobos-Kovács, M., Marialigeti, K., Varga, I., 2000. Morphologic, host specificity, and molecular characterization of a Hungarian *Cryptosporidium meleagridis* isolate. Appl. Environ. Microbiol. 66, 735–8.

Stoskopf, M.K., 1993. Parasites associated with freshwater tropical fishes. In: Stoskopf, M.E. (Ed.). Fish Medicine, Saunders, Philadelphia, PA, pp. 582–3.

Tamburrini, A., Pozio, E., 1999. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis)*. Int. J. Parasitol. 29, 711–5.

Tetley, L., Brown, S.M.A., McDonald, V., Cooms, G.H., 1998. Ultrastructural analysis of the sporozoite of *Cryptosporidium parvum*. Microbiology 144, 3249–55.

Tyzzer, E.E., 1907. A sporozoan found in the peptic glands of the common mouse. Proc. Soc. Exp. Biol. Med. 5, 12–13.

Uni, S., Iseki, M., Maekawa, T., Moriya, K., 1987. Ultrastructure of *Cryptosporidium muris* (strain RN 66) parasitizing the murine stomach. Parasitol. Res. 74, 123–32.

Vetterling, J.M., Takeuchi, A., Madden, P.A., 1971. Ultrastructure of *Cryptosporidium wrairi* from the Guinea pig. J. Protozool. 18, 248–60.

Xiao, L., Morgan, U.M., Fayer, R., Thompson, R.C.A., Lal, A.A., 2000. *Cryptosporidium* systematics and implications for public health. Parasitol. Today 16, 287–92.