4. Benchimol, M. & De Souza, W. 1988. *Tritrichomonas foetus*: freeze-fracture cytochemistry using polymyxin B. *Exp. Parasitol.*, **66**: 35–43.

5. Benchimol, M., Pereira, M. E. A., Elias, C. A. & De Souza, W. 1981. Cell surface carbohydrates in *Tritrichomonas foetus*. *J. Protozool.*, **28**:337–342.

6. Diamond, L. S. 1957. The establishment of various trichomonads of animals and man in axenic cultures. *J. Parasitol.*, **43**:488–490.

7. Gilula, N. B. & Satir, P. 1972. The ciliary necklace. A ciliary membrane specialization. *J. Cell Biol.*, **53**:494–509.

8. Goodenough, U. W. & Heuser, J. E. 1982. Substructure of the outer dynein arm. *J. Cell Biol.*, **95**:798–815.

9. Heuser, J. E. & Kirschner, M. W. 1980. Filament organization revealed in platinum replicas of freeze-dried cytoskeletons. *J. Cell Biol.*, **86**:212–234.

10. Honigberg, B. M. 1978. Tritrichomonads of veterinary importance. *In:* Kreier, J. P. (ed.), Parasitic Protozoa, vol. 2. Academic Press, New York, pp. 164–273.

11. Honigberg, B. M., Mattern, C. F. & Daniel, W. A. 1971. Fine structure of the mastigont systems in *Tritrichomonas foetus*. *J. Protozool.*, **18**:183–198.

12. Honigberg, B. M., Volkmann, D., Entzeroth, R. & Scholtyseck, E. 1984. A freeze-fracture electron microscopic study of *Trichomonas vaginalis* Donné and *Tritrichomonas foetus* (Riedmuller). *J. Protozool.*, **31**:116–131.

Received 12-10-90, 3-18-91; accepted 3-22-91

J. Protozool., 39(4), 1992, pp. 484–494

Life Cycle of *Goussia pannonica* (Molnár, 1989) (Apicomplexa, Eimeriorina), an Extracytoplasmic Coccidium from the White Bream *Blicca bjoerkna*

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ABSTRACT. Life cycle stages of *Goussia pannonica* from naturally-infected white bream *Blicca bjoerkna* were studied by light and electron microscopy. Fourteen of the sixteen fish examined were infected, with developmental stages found in all parts of the intestine. Merogonial, gamogonial, and sporogonial stages were localized intracellularly and extracytoplasmically in the microvillous region of enterocytes. They were separated from the gut lumen by closely apposed enterocyte and parasitophorous vacuole membranes. There were two types of extracytoplasmic attachment: 1) monopodial, with a single zone of attachment, and 2) spider-like, with several isolated zones of attachment to the host cell. First-generation merozoites were formed by ectomerogony. Second- or third-generation merozoites were formed by endodyogeny and endopolygeny. Thirty to 50 biflagellated microgametes developed at the periphery of a microgamont. Macrogamonts contained lipid inclusions, amylopectin and dense granules; however, granules comparable to wall-forming bodies type I and II were absent. At the beginning of sporogony, the sporont cytoplasm detached from two layers which subsequently became constituents of the oocyst wall. After the rupture of enterocyte and parasitophorous vacuole membranes, the sporont was released into the water where exogenous sporulation was completed within 48 h. The thin sporocyst wall contained a small longitudinal suture. Sporocyst and oocyst walls were of similar structure.

Key words. *Cryptosporidium*, enterocyte, fusion, invasion, microvillus, ultrastructure.

As fish coccidia have been given more attention in the last decade, it has become obvious that they differ in many respects from coccidia parasitizing warm-blooded vertebrates [10]. *Cryptosporidium* appears to be the only coccidium inhabiting the microvillous region of the intestine of higher vertebrates [43]. However, in both freshwater and marine fish, the same region is parasitized by a wide spectrum of morphologically different species [22]. In addition to at least two *Cryptosporidium* species [13, 19], one of which considerably extends the range of morphological types within this genus [19], members of three other genera (*Eimeria, Epieimeria,* and *Goussia*) have been found in the microvillous zone of fish intestine [22]. Their developmental stages are localized among the microvilli in the apical part of the enterocyte [17, 20, 22, 27].

Although most members of the genus *Goussia* live intracytoplasmically, *G. acipenseris* [25], *G. girellae* [17], *G. janae* [22], *G. langdoni* [28], and *G. zarnowskii* [15] were recently found in the microvillous region of the intestine. Moreover, *G. spraguei* was described from the microvillous border of kidney tubule epithelium [29].

In his review on coccidiosis in Hungarian fishes, Molnár [26] published a short description of a new species, *G. pannonica,* from the intestine of white bream, *Blicca bjoerkna,* based on light microscopic observations of developmental stages and the morphology of oocysts. This paper presents new data on the ultrastructure of merogonial, gamogonial and sporogonial stages of this interesting parasite.

MATERIALS AND METHODS

Sixteen white bream, *B. bjoerkna,* from Černovický Potok Brook, Soběslav, South Bohemia were sampled in February, March and April 1990 and April and May 1991. Mucosa of the anterior, central and posterior parts of the intestine and the gut contents were examined fresh. Infected tissue was fixed in 10% neutral buffered formalin and paraffin sections were stained with haematoxylin-eosin.

For transmission electron microscopy (TEM), small pieces of intestine were fixed in 2% osmic acid in 0.2 M cacodylate buffer at 4° C for 1 h, dehydrated and embedded in Epon (Serva, Heidelberg)-Araldite (Polysciences, Warrington, PA). Sections were cut with glass knives on an 8800 Ultratome III (LKB, Bromma). Semithin sections were stained with toluidine blue, thin sections were stained either with uranyl acetate and lead citrate [42] or with modified Sato's lead stain solution [11] and examined with a Philips 420 electron microscope. To study exogenous sporulation, feces and fecal casts were incubated in tap water at 20° C for several days. Feces containing sporonts and oocysts were fixed in 2% osmic acid in 0.2 M cacodylate buffer, frozen in this buffer by liquid N_2 for 30 s [3], and then thawed and processed for TEM as described above. Thin sec-

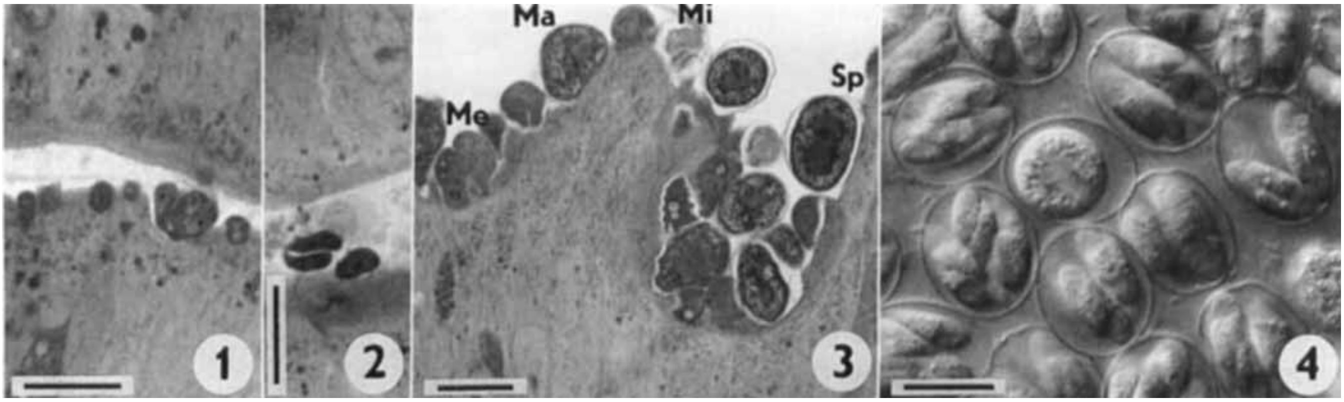


Fig. 1–4. Light microscopy of life cycle stages of *G. pannonica*. Semithin sections stained with toluidine blue (Fig. 1–3) and differential interference contrast optics of fresh material (Fig. 4). **1.** Early meronts and meronts with merozoites formed by ectomerogony. **2.** Two merozoites formed by endodyogeny. **3.** Advanced heavy infection containing meronts (Me), macrogamonts (Ma), microgamonts (Mi) and sporonts (Sp). Note the secondary mucosal folds due to the infection. **4.** Cluster of nearly mature oocysts within casts after 48-h exogenous sporulation. Bars = 10 μm.

tions with oocysts were placed on formvar and carbon-coated grids.

RESULTS

Light microscopy. Of the 16 white breams, 14 were positive for *G. pannonica*. In most cases, the infection was massive and developmental stages were found in all studied segments of the intestine. However, the anterior region was most heavily colonized by the parasite and always harboured the majority of developmental stages. During merogony, early spherical meronts, 3.6–4.6 μm in diameter, exceeded the microvillous region and grew into large irregular meronts, 6.4–10.7 μm × 6.1–7.8 μm. Multiple nuclear division was followed by the formation of 6–12 merozoites (usually eight) by the process of ectomerogony (Fig. 1). Second-generation meronts, undistinguishable by light microscopy from the early meronts, gave rise to two merozoites by endodyogeny (Fig. 2). Both in the early and advanced infections, endodyogenial division was rare; because only natural infections were studied, it was not possible to establish the succession of merogonial generations in the life cycle. All merogonial and gamogonial stages appeared to be attached to the microvillar surface of epithelial cells in an "epicellular" position. Oval macrogamonts (9.7–12.0 μm × 7.2–8.4 μm) contained various irregularly scattered granules that filled the cytoplasm and surrounded the large central nucleus, which contained a prominent nucleolus (Fig. 3). Microgamonts (9.4–12.6 μm × 7.6–9.4 μm) developed from gamonts with less dense homogenous cytoplasm. The number of flagellated microgametes surrounding the large residual body of a mature spherical macrogamont varied from 30 to 50 (Fig. 3).

Large ellipsoidal sporonts (12.2–14.5 μm × 6.5–8.7 μm) detached from the enterocyte and were found in the gut lumen or in feces. No division of the sporont cytoplasm occurred in the intestine. When infections were moderate, sporonts were released in the environment via feces. Heavy infections were manifested by massive discharges of elongated, whitish casts, which were full of sporonts and desquamated enterocytes.

In the merogonial phase the infection resulted in effacement of microvilli with no other visible changes of the infected enterocytes. However, secondary mucosal folds were formed during gamogony and sporogony (Fig. 3).

Sporulation was completely exogenous and sporulated oocysts were observed after 48 h incubation in tap water. The dimensions of oocysts, sporocysts, and sporozoites (Fig. 4) correlated with the data published by Molnár in the original description [26].

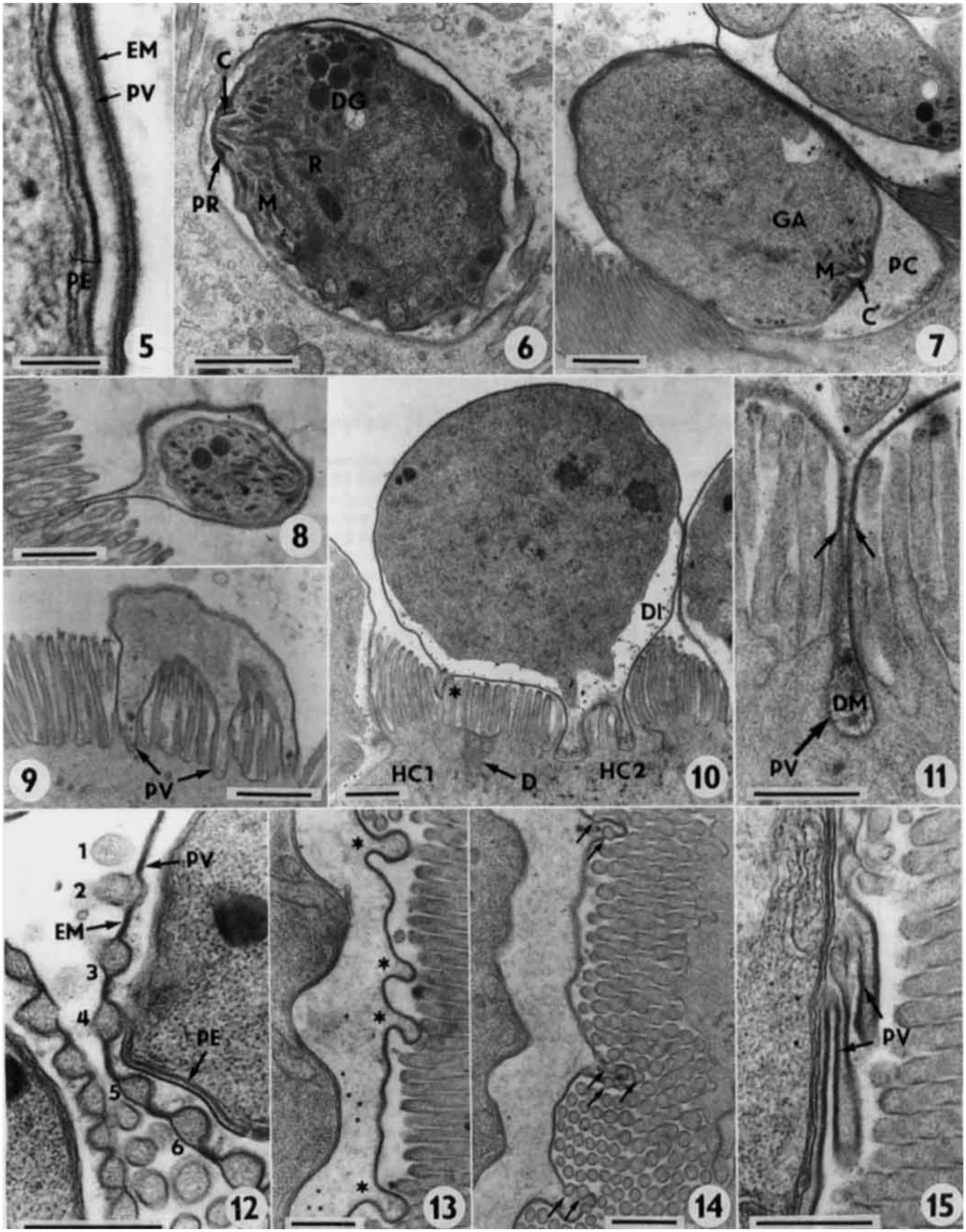
Electron microscopy. Results, obtained from investigation of seven infected fish, showed that all merogonial, gamogonial, and early sporogonial stages were localized in enterocytes, in an intracellular and extracytoplasmic (EC) position. The parasite was separated from the gut lumen by two membranes of host cell origin which were tightly apposed to each other (Fig. 5). The outer membrane was the enterocyte (i.e. microvillous) membrane, and the inner one was the parasitophorous vacuole (PV) membrane. The junction zone between the parasite and the cytoplasm of the enterocyte was covered by a single membrane, the PV membrane (Fig. 6, 7).

Most merogonial and gamogonial stages were attached to the host cell cytoplasm at a single area of contact (Fig. 6, 7, 16, 20, 22, 23, 32). The shape of this area was the result of multiple fusions to microvilli (see below), making it round, irregular, or even an enclosed island of free microvilli (Fig. 29). We assign this location as a "monopodial" EC position.

However, the attachment of many stages differed substantially from the above mentioned type. These stages were localized above the microvillous zone, connected with the intestinal epithelium only through the mediation of several thin intravillar projections, in a "spider-like" arrangement (Fig. 8–10, 14, 17, 33). These stages were also covered on their surfaces by double membranes—the enterocyte and PV membranes; however, in the several small areas of contact with the enterocyte cytoplasm they were separated by the PV membrane only (Fig. 9, 10, 33).

On the transverse sections of the periphery of some growing stages, the multiple fusions of several neighbouring microvilli with the enterocyte membrane covering the parasite occurred (Fig. 12). In the course of fusion, the fibrillar content of the fused microvilli was drawn in between the enterocyte and PV membranes, making the latter membrane more twisted than the former one (Fig. 12). Finally, the close apposition of both membranes was restituted. Although the monopodial or spider-like status of individual stages could be reliably distinguished only on oblique sections, these stages were probably the monopodial ones.

On the periphery of other stages, transverse sections showed various projections of both enterocyte and PV membranes (Fig. 13). Fusions to microvilli, of the same type as described above, occurred only on the tips of these projections, the fusion sites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being therefore apart from each other (the probable spider-like stages) (Fig. 14, 15).

In oblique sections of the intravillous projections of spider-like stages (Fig. 11, 15), the PV membrane was closely apposed to the enterocyte membrane in the lumen of the villus and penetrated to the base of the villus where it came into contact with the host cell cytoplasm (Fig. 9, 11). Electron-dense material was scattered in the PV content of these projections (Fig. 11, 33). The fusion of limiting membranes of two neighbouring enterocytes via a large spider-like stage, which was in fact intracellular for two host cells (HC 1 and HC 2), was occasionally observed (Fig. 10). The ultrastructural features of spider-like meronts did not differ from those of monopodial ones.

Small round meronts with a trimembraneous pellicle (Fig. 5), probably sporozoite-derived, contained several rhoptries, dense granules, short numerous micronemes, a polar ring, a conoid with two preconoidal rings, a microporus, a nucleus, mitochondria, and small vacuoles (Fig. 6). Only these stages, not reaching above the microvilli, were present in the early phase of infection. More advanced meronts were characterized by abundant endoplasmic reticulum and Golgi apparatus, and by a persistent conoid which was directed toward the host cell cytoplasm and was present until the merogonial division, and accompanied by peripherally scattered remnants of micronemes (Fig. 7). Meronts were rarely situated in the apices of goblet cells (Fig. 20).

Following multiple nuclear division of the meront, the parasite membrane began to invaginate. Up to 12 merozoites developed by ectomerogony detached from the residual cytoplasm and appeared in a random bundle (Fig. 16–18). After the newly-formed merozoites, which contained all typical organelles, were released following rupture of both the enterocyte and PV membranes (Fig. 21), regions effaced of microvilli remained (Fig. 19). Rarely, merozoites formed by endodyogeny and endopolygeny have been observed. They originated from second (?) or third (?) generation meronts which differed from the early ones by the persistence of oval dense granules in their cytoplasm (Fig. 22). After the merozoite anlagen developed in the mother cell (Fig. 23–25), which was full of micronemes and dense granules, two stout merozoites were subsequently formed by the process of endodyogeny (Fig. 26). In other meronts, very rare in our material, endopolygenial division led to the formation of at least four merozoites (Fig. 27, 28). The meronts which gave rise to gamonts substantially increased in size and protruded into the lumen. The multiple division of nuclei was characteristic for young microgamonts (Fig. 29). The microgametes that developed at the periphery of mature microgamonts consisted of a perforatorium, two flagella, a mitochondrion, and a dense elongate nucleus (Fig. 29, 30).

Young macrogamonts contained large lipid inclusions and round electron-dense bodies scattered unequally in the cytoplasm (Fig. 31). They had a centrally located nucleus with a conspicuous nucleolus. Further development was accompanied by the appearance of small amylopectin granules which, in mature macrogamonts, significantly increased in size and number, whereas lipid inclusions gradually dwindled. No evidence of direct contact between the macrogamont and enterocyte via the intravacuolar blebs or tubules was observed. In sections of merogonial and gamogonial stages of *G. pannonica*, small electron-dense inclusions, 20 to 50 nm in diameter, were frequently observed. They were unevenly distributed in the parasite cytoplasm, cytoplasmic vacuoles, and in the PV content (Fig. 6, 10, 21, 29, 31, 33). They were not observed outside the PV in the enterocyte or in the gut lumen. While their abundance was obviously not stage dependent, significant variations occurred in sections of individual parasitic cells.

The early phase of sporogony continued in the EC position. Although most gamogonial and sporogonial stages were observed in a monopodial position (Fig. 31, 32), some stages were attached to the host cell by spider-like projections (Fig. 33). The examination of young ellipsoidal sporonts with the peripheral cytoplasm full of amylopectin and dense granules (Fig. 33) showed

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Fig. **5–15**. Ultrastructure of merogonial stages of *G. pannonica*. **5**. Closely apposed enterocyte cytoplasmic membrane (EM) and parasitophorous vacuole membrane (PV) with underlying trimembraneous pellicle (PE) of the coccidium. Bar = 0.1 μm. **6**. Early meront containing conoid (C), polar ring (PR), rhoptries (R), micronemes (M), and dense granules (DG). Bar = 1 μm. **7**. Meront during dedifferentiation; components of the apical complex have disappeared except for the conoid (C) and peripherally scattered micronemes (M). GA, Golgi apparatus; PC, parasitophorous vacuole content. Bar = 1 μm. **8**. Early meront protruding above the microvillous region. Bar = 1 μm. **9**. Spider-like meront. PV, parasitophorous vacuole membrane. Bar = 1 μm. **10**. Young multinucleate spider-like meront fixed by several zones of attachment to two host cells (HC 1 and HC 2) in this section. *, projection; D, desmosome. This stage is virtually intracellular at the same time for at least two host cells. Note numerous small electron-dense inclusions (DI) in the parasitophorous vacuole. Bar = 1 μm. **11**. Parasitophorous vacuole membrane (PV) penetrates to the base of the fused microvillus and is in contact with the host cell cytoplasm. Parasitophorous vacuole projection contains amorphous electron-dense material (DM). Arrows, site of the close apposition of parasitophorous vacuole and enterocyte membranes. Bar = 0.5 μm. **12–15**. Transverse and oblique sections through the periphery of growing meronts. **12**. Multiple fusions of individual microvilli to the enterocyte membrane surrounding the coccidium. Follow the sequence (1–6) of this event during which the fibrillar content of microvilli is indrawn between the enterocyte (EM) and parasitophorous vacuole (PV) membranes, making the latter membrane twisted. PE, pellicle. Bar = 0.5 μm. **13**. Small projections (*) of enterocyte and parasitophorous vacuole membranes coming into contact with microvilli. Bar = 0.5 μm. **14**. Cluster-like fusions (arrows) of microvilli to the enterocyte membrane on the tips of projections (the establishment of spider-like position). Bar = 0.5 μm. **15**. Oblique section through two adjacent projections. Note that the parasitophorous vacuole membrane (PV) has already penetrated into the lumen of the fused microvilli. Bar = 0.5 μm.

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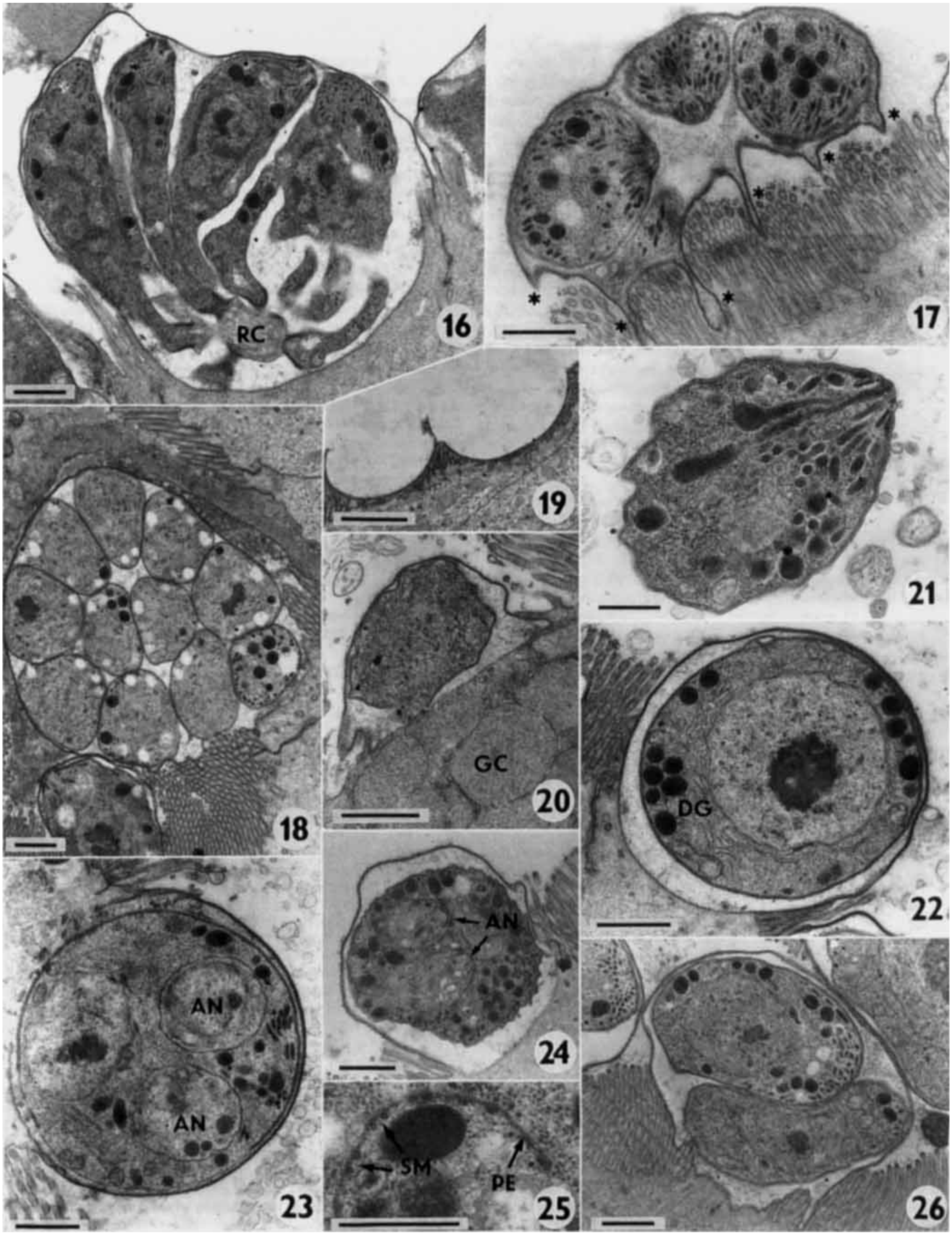
Fig. **16–26**. Ultrastructure of merogonial stages of *G. pannonica*. **16**. Merozoites formed by ectomerogony. RC, residual cytoplasm. Bar = 1 μm. **17**. Spider-like meront containing mature merozoites. Note attachment to the enterocyte by at least six projections (*) in this section. Bar = 1 μm. **18**. Numerous mature merozoites in transverse section through meront. Bar = 1 μm. **19**. Effaced regions of microvilli after the detachment of merogonial stages. Bar = 2 μm. **20**. Meront localized in the apical part of a goblet cell (GC). Bar = 1 μm. **21**. Merozoite free in the gut lumen prior to the host cell invasion. Bar = 0.5 μm. **22**. Oval, second (?) generation meront with numerous dense granules (DG) in the cytoplasm. Bar = 1 μm. **23, 24**. Transverse and longitudinal sections through merozoites during their formation by endodyogeny in the meront cytoplasm; AN, merozoite anlagen. Bar = 1 μm. **25**. Detail of the merozoite anlagen with pellicle (PE) and subpellicular microtubules (SM). Bar = 0.5 μm. **26**. Two stout merozoites formed by endodyogeny. Bar = 0.5 μm.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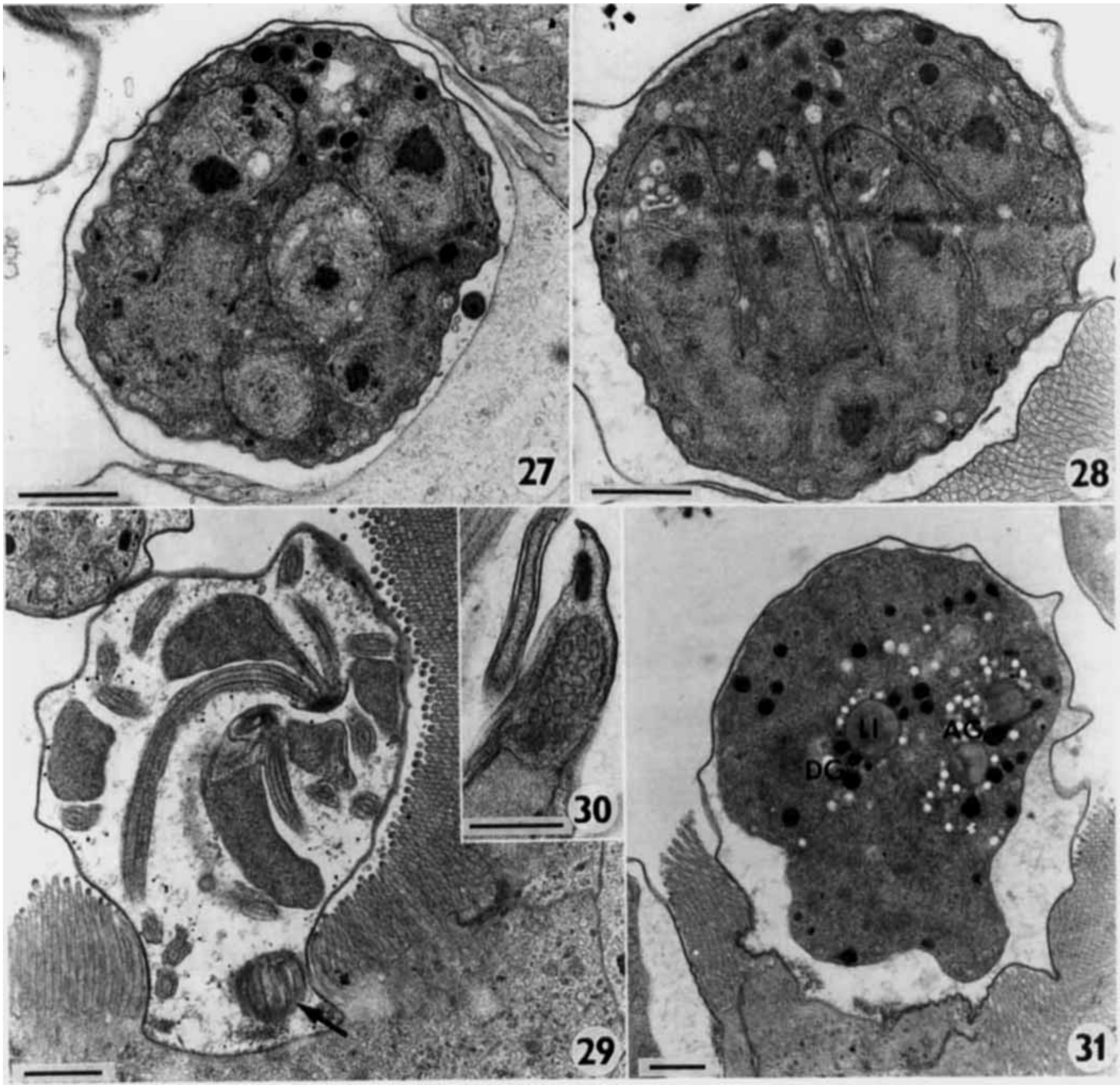

Fig. 27–31. Ultrastructure of *G. pannonica*. **27**, **28**. Merogonial stages. **29–31**. Gamogonial stages. **27**. Early stage of endopolygeny. Bar = 1 μm. **28**. Merozoites formed by endopolygeny. Bar = 1 μm. **29**. Microgametocyte containing mature biflagellate microgametes. The zone of attachment encloses an island of free microvilli (arrow). Bar = 1 μm. **30**. Prominent perforatorium of a mature microgamete. Bar = 0.5 μm. **31**. Early macrogametocyte containing lipid inclusions (LI), amylopectin granules (AG) and dense granules (DG). Bar = 1 μm.

the formation of several superimposed layers which detached from the sporont cytoplasm. At its periphery, multiple concentric layers of rough endoplasmic reticulum were present (Fig. 34).

Oocyst wall formation began with the detachment of two independent layers (Fig. 34) which were closely apposed to each other during further development (Fig. 35). Shortly before the rupture of enterocyte and PV membranes, the layers of the future sporocyst wall were formed; however, they did not detach from the sporont cytoplasm (Fig. 34, 35). During the exogenous part of the life cycle, the formation of the oocyst wall was followed by the deposition of electron-dense material under the inner layer (Fig. 39).

Cleavage of the sporont cytoplasm resulted in the formation of two, and subsequently four, sporoblasts. In each sporoblast only a nucleus, crystalloid body (Fig. 38), amylopectin granules and lipid inclusions were visible. The sporocyst wall was of uniform thickness (about 20 nm) (Fig. 36), except where it gave rise to the longitudinal suture where it was about 70 nm thick (Fig. 37). The wall was composed of an electron-dense, inner layer (about 14 nm) lying beneath a thin, electron-lucent layer (about 6 nm) covered by an electron-dense coating of variable

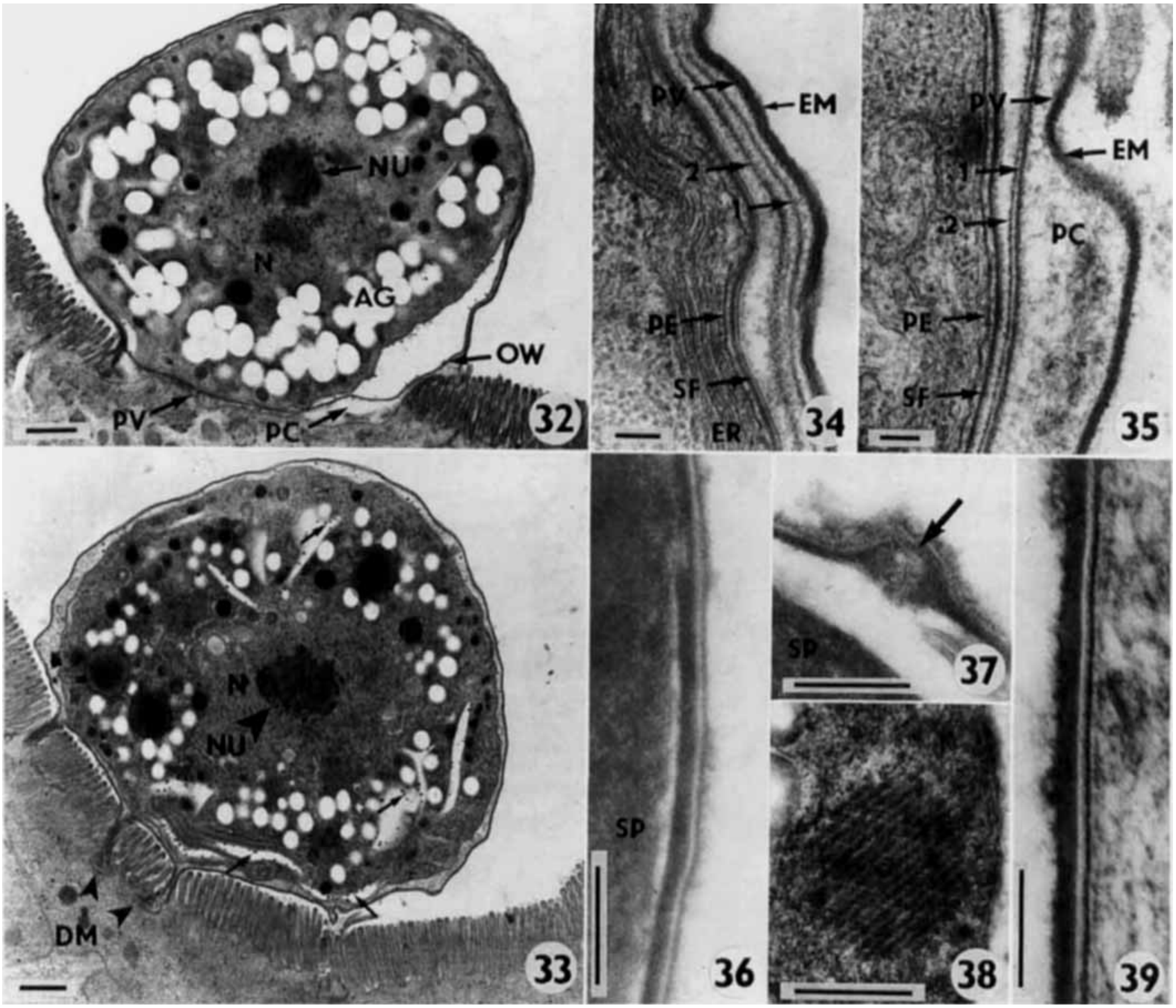

Fig. 32-39. Ultrastructure of *G. pannonica*. **32-35**. Endogenous sporogonial stages. **36-39**. Exogenous sporogonial stages. **32**. Sporont in a monopodial extracytoplasmic location containing numerous amylopectin granules (AG). The oocyst wall (OW) is detached from the sporont cytoplasm. PC, parasitophorous vacuole content; PV, parasitophorous vacuole membrane; N, nucleus; NU, nucleolus. Bar = 1 μm. **33**. Early spider-like sporont attached to the enterocyte by four projections in this section. Note that plane of the section leads through the centrally located nucleus (N) and nucleolus (NU). DM, dense material in the parasitophorous vacuole content of the projections; arrows, dense inclusions. Bar = 1 μm. **34**. Periphery of mature sporont, hitherto in extracytoplasmic location. Foundations of the sporocyst wall have already formed (SF) and two independent layers (1, 2) are detached from the sporont cytoplasm. Note the presence of multiple concentric layers of endoplasmic reticulum (ER) under pellicle (PE). EM, enterocyte membrane; PV, parasitophorous vacuole membrane. Bar = 0.1 μm. **35**. Periphery of mature sporont, more advanced than the stage in Fig. 34, prior to rupture of the enterocyte (EM) and parasitophorous vacuole membranes (PV). Two independent layers (1, 2) are closely apposed upon each other and form the foundation of the oocyst wall. PC, parasitophorous vacuole content. Bar = 0.1 μm. **36**. Sporocyst wall of the sporulated oocyst. SP, sporozoite. Bar = 0.1 μm. **37**. Transverse section through the suture (arrow) of the sporocyst wall. SP, sporozoite. Bar = 0.1 μm. **38**. Crystalloid body in the sporozoite cytoplasm. Bar = 0.5 μm. **39**. Oocyst wall of the sporulated oocyst. Bar = 0.1 μm.

thickness (Fig. 36). The longitudinal suture divided the sporocyst wall into two identical shelves (Fig. 37). The oocyst and sporocyst walls were of similar structure. The oocyst wall was also composed of three layers, the inner layer being thickest (25 nm). The central, electron-lucent layer was superimposed by an outer dense layer of similar thickness (about 6–8 nm) (Fig. 39).

DISCUSSION

Ultrastructural studies have shown that the individual stages, which by light microscopy appeared to be in an epicellular position, were in fact localized under the enterocyte membrane in a PV, i.e. in an intracellular position. However, the PV membrane surrounding the coccidium was apposed to the enterocyte membrane so that contact with the host cell cytoplasm was restricted to a small area. Therefore we would designate this type of localization as EC. In contrast to *Cryptosporidium* sp. [24], there was no cytoplasmic rim sandwiched between the enterocyte and PV membranes.

According to recent investigations of fish coccidia [7, 17, 20, 22, 25, 26, 28, 29] (Lukeš, J. 1990. Freshwater fish coccidia: species composition, biology and ultrastructure. Ph.D. dissertation, Czech Acad. Sci.) it has become obvious that EC species represent a much larger and more common group of species than previously expected. It seems that in most studies of fish parasites the EC coccidia have been regularly overlooked for three primary reasons: 1) in contrast to most fish coccidia, these species sporulate exogenously; 2) in fresh material the small developmental stages can only be detected with difficulty; 3) most EC species exhibit a strict seasonal occurrence (spring in central Europe).

All but two of the white bream examined were infected, with most harbouring heavy infections. Given our previous work with *G. janae* [22], it is likely that these two specimens were negative for infection because of high water temperature (20° C) during their prolonged stay in the laboratory. Infected fish exhibited no visible signs of infection, although, especially during gamogony and sporogony, their intestinal epithelium was considerably altered by the effacement of microvilli and deep secondary mucosal folds followed by desquamation.

The EC location has been described in *Cryptosporidium* sp. which possessed a distinct attachment organelle, probably of parasitic origin [5, 30], which enables the intimate contact with the enterocyte cytoplasm.

Morphological structures similar to the feeder organelle have not been observed in *Epieimeria* species [6, 27] or in EC members of the genera *Eimeria* and *Goussia* [15, 20, 29], including *G. pannonica.* The arrangement of the PV membrane was species specific based on various projections and undulations; however, there was no direct contact between the parasite and the host cell cytoplasm.

Although numerous early merogonial stages have been examined, the parasite's entry into the enterocyte has not been traced. Sporozoite attachment which is accompanied by the formation of thin, host-derived cytoplasmic extensions which eventually enclose the parasite in the PV [23, 24, 30] as in *Cryptosporidium* could probably also occur in zoite invasion by *G. pannonica* (Fig. 40). Moreover, the possible general distribution of this mechanism among EC coccidia is supported by observations made on merogonial stages of *Eimeria* sp. from geckoes [31] which are ultrastructurally quite similar to the same stages of *G. pannonica.* The short duration of this event [23] makes it difficult to observe in natural infections. In our material, two stages with different types of EC location, termed monopodial and spider-like, were found. The former, more common stage was similar to the EC stages described in other EC coccidia [15, 27, 29]. The spider-like stage was localized above the microvillous border and connected with the enterocyte by multiple thin projections.

Paperna [31] and Paperna and Landsberg [32] described stages "connected with the host cell through rootlet-like connections" which occurred during the life cycle of an intestinal EC coccidium from geckoes. They considered the individual connections to be cross sections through deeply infolded sides of PV of this parasite, for which they constituted a new genus *Acroeimeria.* My scanning electron microscope study (Lukeš, unpubl. data) and analysis of many consecutive sections has shown that this is not the case in the spider-like stages of *G. pannonica.*

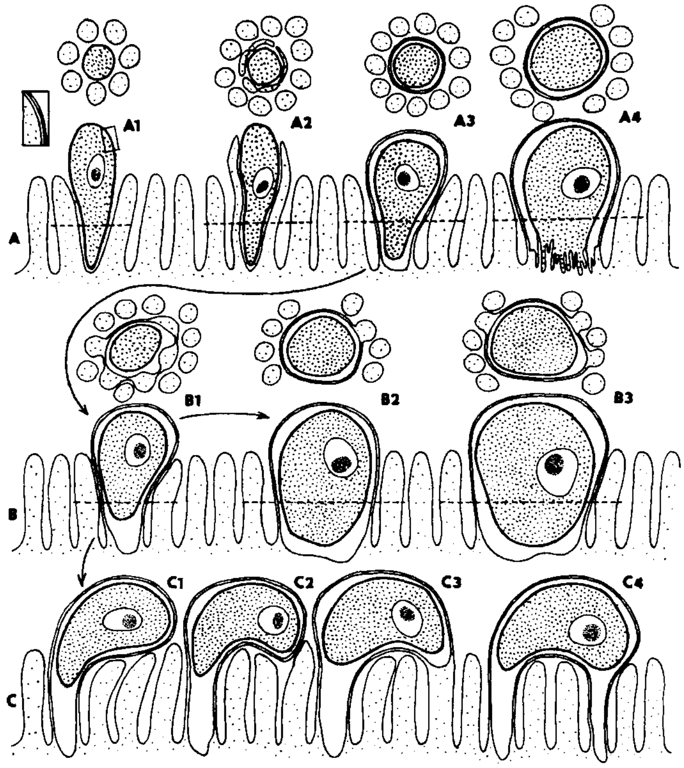

Fig. **40**. Diagrammatic interpretation of host cell invasion. **A**. Invasion by *Cryptosporidium* zoites (schematized according to [5, 23, 24, 30 and 39]). **B**, **C**. Invasion by *G. pannonica* zoites. **A1**. Zoite penetration among microvilli. **A2**. Cytoplasmic extensions of surrounding microvilli. **A3**. Enclosure of the coccidium by surrounding microvilli in intracellular and extracytoplasmic location. **A4**. Formation of the attachment organelle and subsequent growth of the coccidium. **B**. Monopodial extracytoplasmic location. **B1**. Following A1–3. Multiple fusions of surrounding microvilli with the enterocyte membrane investing the coccidium. **B2**. Subsequent extension of contact area with the host cell cytoplasm. **B3**. Growth of the parasite leading to other lateral fusions. **C**. Spider-like extracytoplasmic location. **C1**. Following A1–A3, B1. Extention of the coccidium above the microvillous region. **C2**. Establishment of contact with microvilli apart from the primary zone of attachment. **C3**. Fusion of the enterocyte membrane surrounding the coccidium to these microvilli. **C4**. Establishment of a new contact with the host cell cytoplasm by penetration of the parasitophorous vacuole membrane to the base of fused microvilli.

Interesting observations were recently made on gamonts of *Haemogregarina balli* from leeches, which when free in the gut lumen are surrounded by an extracellular sheath comprised of two closely apposed membranes. The EC position with several zones of attachment was achieved by the fusion of microvilli to the membranous sheath of the gamont [36]. Thus, both membranes separating the coccidium from the lumen are of parasite origin.

This feature represents a principal difference from the host-parasite system of *G. pannonica.* The spider-like position of merogonial and gamogonial stages of *G. pannonica* was achieved by the fusion of enterocyte membrane to individual microvilli which were situated various distances (even in another enterocyte, Fig. 10) from the primary zone of attachment (Fig. 40). This was in fact fusion between several microvilli, some of which were extremely enlarged by the developing parasite. The process was followed by the close apposition of the PV membrane under

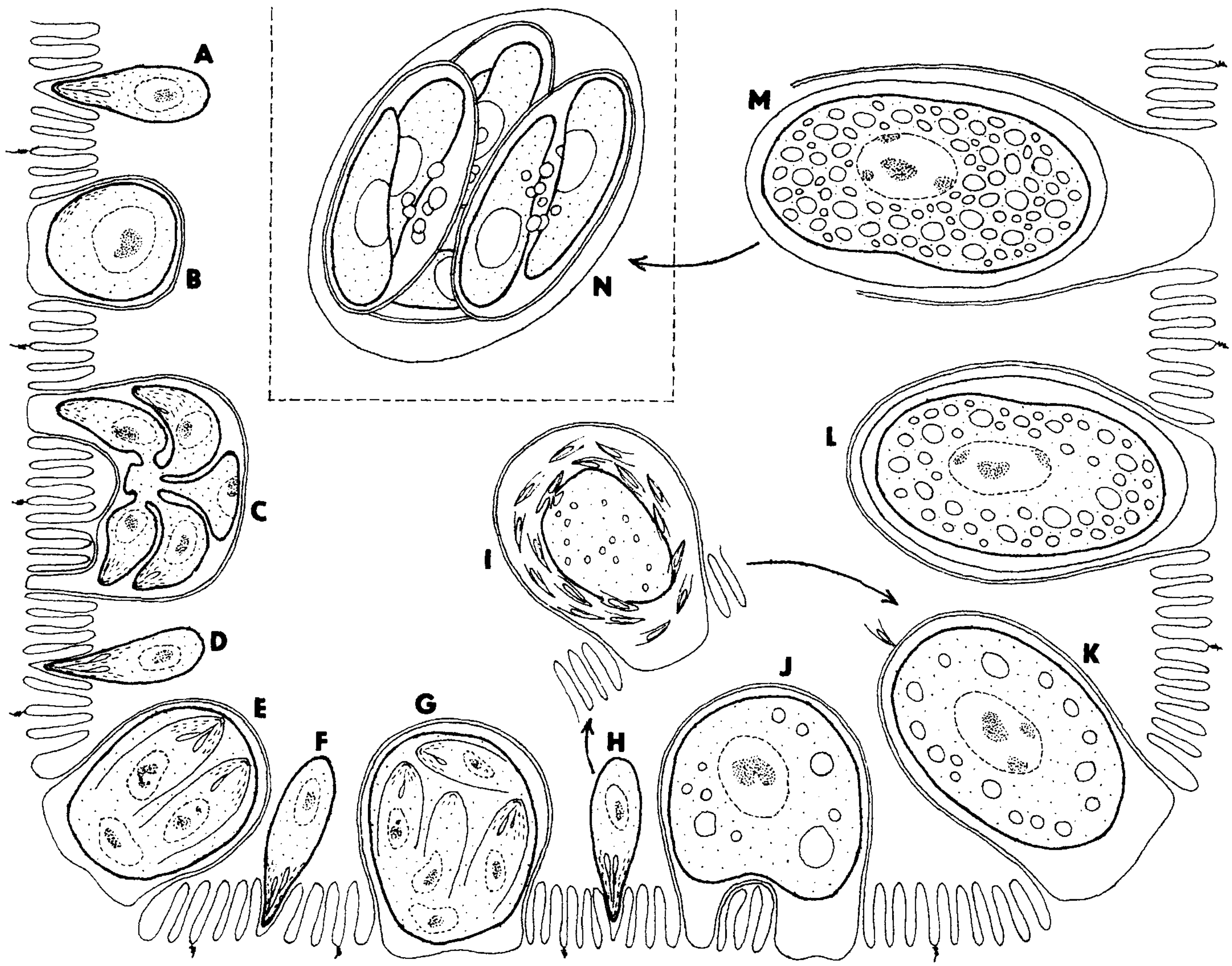

Fig. 41. Diagrammatic representation of the development of *G. pannonica* in the intestine of the white bream, *B. bjoerkna*. **A.** Zoite invasion in an enterocyte. **B.** Large oval meront. **C.** First-generation merozoites formed by ectomerogony in spider-like meront. **D, F, H.** Merozoites invading the enterocytes. **E.** Second-generation merozoites formed by endodyogeny in monopodial meront. **G.** Third-generation merozoites formed by endomerogony in monopodial meront. **I.** Microgametocyte with the biflagellate microgametes at the periphery. **J.** Young macrogametocyte. **K.** Zygote containing several lipid inclusions, amylopectin, and dense granules. **L.** Sporont containing multiple amylopectin granules. The oocyst wall is already formed. **M.** The rupture of enterocyte and parasitophorous vacuole membranes and the release of sporont in the gut lumen. **N.** Sporulated oocyst after 48-h exogenous sporulation in tap water.

the membrane of the newly fused microvillus and its penetration at the base of the microvillus where the membrane came into contact with the enterocyte cytoplasm (Fig. 40). The consequence of this event, whose mechanism remains unclear, was the existence of parasite stages localized above the microvillous border, which were virtually in intracellular and EC position, fixed to the host cell by several discrete regions. The establishment of an intracellular location of a coccidium in or above the microvillous border, involving the multiple fusion of neighbouring or separate microvilli, has not been described previously. All spider-like stages developed from earlier monopodial ones (Fig. 40); however, it is obvious that at least part of gamogonial and sporogonial stages remained in monopodial localization throughout the intracellular phase of the life cycle.

A diagrammatic representation of the development of *G. pannonica* in the intestine of *B. bjoerkna* is presented in Fig. 41.

The observation of a persistent conoid which was usually found in cyst-forming coccidia [4] corresponds with observations made on another EC species, *G. zarnowskii* [15]. In fish coccidia, both ectomerogonial [2, 14, 22, 40] and endomerogonial [27, 34, 37] divisions have been found. A third type of merogonial division, endodyogeny, is less frequent. It has been described only in *Eimeria vanasi* [20], *Eimeria* sp. from *Atherinomorphus capricornensis* [18] and EC *Goussia* sp. from *Gobio gobio* (Lukeš, unpubl. data).

The involvement of two different types of merogonial division in one life cycle has been published for only two fish coccidia: *E. vanasi* [20] and *G. iroquina* [34]. However, the combination of ectomerogony, endomerogony (endopolygeny) and endodyogeny as it is described herein for the life cycle of *G. pannonica* is unique, not only among fish coccidia, but apparently among coccidia in general.

Microgametes developing in limited numbers on the periphery of microgamonts possessed two flagella. The existence of aflagellated microgametes in *Cryptosporidium* sp. [5, 30, 39], unique among higher coccidia, was explained by Barta [1] as an adaptation to the highly exposed (=EC) location of macrogamonts not requiring motile microgametes. The presence of flagella in microgametes of *G. pannonica* and the EC members of the genera *Eimeria, Epieimeria* and *Goussia* from fish [15, 22, 27] is at variance with this opinion. Considering the lack of feeder organelles in this species, it may, however, reflect their closer relationship to eimeriorins rather than to *Cryptosporidium* which, according to rRNA sequences is relatively distant from other apicomplexans [16].

We have no satisfactory explanation for the occurrence of small round dense inclusions present in the parasite cytoplasm, cytoplasmic vacuoles and PV content. They were probably of artificial origin.

Numerous amylopectin granules and a limited number of lipid inclusions and dense granules were present in the cytoplasm of macrogamonts and young sporonts. These granules, however, did not appear to participate in the formation of the oocyst wall. Other granules, comparable to wall-forming bodies of types I and II described in various eimerian coccidia [4], were absent. This is in agreement with observations on most fish coccidia [8, 9, 38], although several authors have described structures similar to wall-forming bodies [12, 33]. Before the rupture of both enterocyte and PV membranes, while still in the EC location, two layers detached independently from the sporont and dense layers of the future sporocyst wall were formed. We suppose that the oocyst wall, being thicker than the sporocyst wall, was formed by close application of both layers upon each other and by subsequent deposition of the dense material under the inner layer. The sporocyst wall and the longitudinal suture that lacked membranaceous veils were significantly thinner but otherwise were identical structurally to most intracytoplasmic and EC members of the genus *Goussia* [2, 21, 29, 35]; however, species having the sporocyst wall thinner than the oocyst wall are rare among fish coccidia [17, 41].

ACKNOWLEDGMENTS

I thank Drs. Jiří Lom and Iva Dyková for their continuous support and for reading the manuscript, and the latter for her help with light micrographs. The help of Ing. Tomáš Kepr in collecting the material is acknowledged.

LITERATURE CITED

1. Barta, J. R. 1989. Phylogenetic analysis of the class Sporozoea (Phylum Apicomplexa Levine 1970): evidence for the independent evolution of heteroxenous life cycles. *J. Parasitol.*, **75**:195–206.

2. Baska, F. & Molnár, K. 1989. Ultrastructural observations on different developmental stages of *Goussia sinensis* (Chen, 1955), a parasite of the silver carp (*Hypophthalmichthys molitrix* Valenciennes, 1844). *Acta Vet. Hung.*, **37**:81–87.

3. Beesley, J. E. & Latter, V. S. 1982. The sporulation of *Eimeria tenella* as revealed by a novel preparative method. *Z. Parasitenkd.*, **67**: 255–260.

4. Chobotar, B. & Scholtysek, E. 1982. Ultrastructure. *In:* Long, P. L. (ed.), The Biology of the Coccidia. University Park Press, London, pp. 101–167.

5. Current, W. L. & Reese, N. C. 1986. A comparison of endogenous development of three isolates of *Cryptosporidium* in suckling mice. *J. Protozool.*, **33**:98–108.

6. Daoudi, F., Marquès, A. & Bouix, G. 1985. Ultrastructure d'*Epieimeria isabellae* Lom and Dyková, 1982, coccidie épicellulaire parasite intestinal du poisson téléostéen *Conger conger* Linné, 1758. *J. Protozool.*, **32**:A114.

7. Daoudi, F., Radujkovič, B., Marquès, A. & Bouix, G. 1987. Nouvelles espèces de Coccidies (Apicomplexa, Eimeriidae) des genres *Eimeria* Schneider, 1875, et *Epieimeria* Dykova et Lom, 1981, parasites de poissons marins de la baie de Kotor (Yougoslavie). *Bull. Mus. Natn., Hist. Nat. Paris*, **9**:321–332.

8. Davies, A. J. 1990. Ultrastructural studies on the endogenous stages of *Eimeria variabilis* (Thélohan, 1893) Reichenow, 1921, from *Cottus (Taurulus) bubalis* Euphrasen (Teleostei: Cottidae). *J. Fish Dis.*, **13**:447–461.

9. Desser, S. S. & Li, L. 1984. Ultrastructural observations on the sexual stages and oocyst formation in *Eimeria laureleus* (Protozoa, Coccidia) of perch, *Perca flavescens*, from Lake Sasajewun, Ontario. *Z. Parasitenkd.*, **70**:153–164.

10. Dyková, I. & Lom, J. 1981. Fish coccidia: critical notes on life cycles, classification and pathogenicity. *J. Fish Dis.*, **4**:487–505.

11. Hanaichi, T., Sato, T., Iwamoto, T., Malavashi-Yamamoto, J., Hoshino, M. & Mizuno, N. 1986. A stable lead by modification of Sato's method. *J. Electron Microsc.*, **35**:304–306.

12. Hawkins, W. E., Solangi, M. A. & Overstreet, R. M. 1983. Ultrastructure of the macrogamont of *Eimeria funduli*, a coccidian parasitizing killifishes. *J. Fish Dis.*, **6**:33–43.

13. Hoover, D. M., Hoerr, F. J., Carlton, W. M., Hinsman, E. J. & Ferguson, H. W. 1981. Enteric cryptosporidiosis in a naso tang, *Naso lituratus* Bloch and Schneider. *J. Fish Dis.*, **4**:425–428.

14. Jastrzebski, M. 1989. Ultrastructural study on the development of *Goussia aculeati*, a coccidium parasitizing the three-spined stickleback, *Gasterosteus aculeatus*. *Dis. Aquat. Org.*, **6**:45–53.

15. Jastrzebski, M. & Komorowski, Z. 1990. Light and electron microscopic studies on *Goussia zarnowskii* (Jastrzebski, 1982), an intestinal coccidium parasitizing the three-spined stickleback, *Gasterosteus aculeatus*. *J. Fish Dis.*, **13**:1–24.

16. Johnson, A. M., Fielke, R., Lumb, R. & Baverstock, P. R. 1990. Phylogenetic relationships of *Cryptosporidium* determined by ribosomal RNA sequence comparison. *Int. J. Parasitol.*, **20**:141–147.

17. Kent, M. L., Fournie, J. W., Snodgrass, R. E. & Elston, R. A. 1988. *Goussia girellae* n. sp. (Apicomplexa, Eimeriorina) in the opaleye, *Girella nigricans*. *J. Protozool.*, **35**:287–290.

18. Kent, M. L., Moser, M. & Fournie, J. W. 1989. Coccidian parasites (Apicomplexa: Eucoccidiorida) in hardy head fish, *Atherinomorphus capricornensis* (Woodland). *J. Fish Dis.*, **12**:179–183.

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

20. Landsberg, J. H. & Paperna, I. 1987. Intestinal infections by *Eimeria* (s.l.) *vanasi* n. sp. (Eimeriidae, Apicomplexa, Protozoa) in cichlid fish. *Ann. Parasitol. Hum. Comp.*, **62**:283–293.

21. Lom, J. 1971. Remarks on the spore envelopes of fish coccidia. *Folia Parasitol.*, **18**:289–293.

22. Lukeš, J. & Dyková, I. 1990. *Goussia janae* n. sp. (Apicomplexa, Eimeriorina) in dace *Leuciscus leuciscus* and chub *L. cephalus*. *Dis. Aquat. Org.*, **8**:85–90.

23. Lumb, R., Smith, K., O'Donoghue, P. J. & Lanser, J. A. 1988. Ultrastructure of the attachment of *Cryptosporidium* sporozoites to tissue culture cells. *Parasitol. Res.*, **74**:531–536.

24. 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**:583–594.

25. Molnár, K. 1986. Occurrence of two new *Goussia* species in the intestine of the sterlet (*Acipenser ruthenus*). *Acta Vet. Hung.*, **34**: 169–174.

26. Molnár, K. 1989. Nodular and epicellular coccidiosis in the intestine of cyprinid fishes. *Dis. Aquat. Org.*, **7**:1–12.

27. Molnár, K. & Baska, F. 1986. Light and electron microscopic studies on *Epieimeria anguillae* (Léger et Hollande, 1922), a coccidium parasitizing the european eel, *Anguilla anguilla* L. *J. Fish Dis.*, **9**:99–110.

28. Molnár, K. & Rohde, K. 1988. New coccidian from freshwater fishes of Australia. *J. Fish Dis.*, **11**:161–169.

29. Morrison, C. M. & Poynton, S. L. 1989. A new species of *Goussia* (Apicomplexa, Coccidia) in the kidney tubules of the cod, *Gadus morhua* L. *J. Fish. Dis.* **12**:533–561.

30. Ostrovska, K. & Paperna, I. 1990. *Cryptosporidium* sp. of the starred lizard *Agama stellio*: ultrastructure and life cycle. *Parasitol. Res.*, **76**:712–720.

31. Paperna, I. 1989. Ultrastructure of *Eimeria* (s.l.) sp. infecting the microvillar zone of the intestinal epithelium of geckoes. *Ann. Parasitol. Hum. Comp.*, **64**:89–99.

32. Paperna, I. & Landsberg, J. H. 1989. Description and taxonomic discussion of eimerian coccidia from African and Levantine geckoes. *S. Afr. J. Zool.*, **24**:345–355.

33. Paperna, I., Landsberg, J. H. & Feinstein, N. 1986. Ultrastructure of the macrogamont of *Goussia cichlidarum* Landsberg and Paperna, 1985, a coccidian parasite in the swimbladder of cichlid fish. *Ann. Parasitol. Hum. Comp.*, **61**:511–520.

34. Paterson, W. B. & Desser, S. S. 1981. An ultrastructural study of *Eimeria iroquina* Molnár and Fernando, 1974 in experimentally infected fathead minnows (*Pimephales promelas*, Cyprinidae). 3. Merogony. *J. Protozool.*, **28**:302–308.

35. Paterson, W. B. & Desser, S. S. 1984. Ultrastructural observations on fertilization and sporulation in *Goussia iroquina* (Molnár and Fernando, 1974) in experimentally infected fathead minnows (*Pimephales promelas*, Cyprinidae). *J. Parasitol.*, **70**:703–711.

36. Siddall, M. E. & Desser, S. S. 1990. Gametogenesis and sporogonic development of *Haemogregarina balli* (Apicomplexa: Adeleida: Haemogregarinidae) in the leech *Placobdella ornata*. *J. Protozool.*, **37**:511–520.

37. Steinhagen, D. 1991. Ultrastructural observations on merogonic and gamogonic stages of *Goussia carpelli* (Apicomplexa, Coccidia) in experimentally infected common carp *Cyprinus carpio*. *Europ. J. Protistol.*, **27**:71–78.

38. Steinhagen, D., Lukeš, J. & Körting, W. 1990. Ultrastructural observations on gamogonic stages of *Goussia subepithelialis* (Apicomplexa, Coccidia) from common carp *Cyprinus carpio*. *Dis. Aquat. Org.*, **9**:31–36.

39. Uni, S., Iseki, M., Maekawa, T., Moriya, K. & Takada, S. 1987. Ultrastructure of *Cryptosporidium muris* (strain RN 66) parasitizing the murine stomach. *Parasitol. Res.*, **74**:123–132.

40. Upton, S. J. & Duszynski, D. W. 1982. Development of *Eimeria funduli* in *Fundulus heteroclitus*. *J. Protozool.*, **29**:66–71.

41. Upton, S. J., Gardner, S. L. & Duszynski, D. W. 1988. The round stingray, *Urolophus halleri* (Rajiformes: Dasyatidae), as a host for *Eimeria chollaensis* sp. nov. (Apicomplexa: Eimeriidae). *Can. J. Zool.*, **66**:2049–2052.

42. Venable, J. H. & Coggeshall, R. 1965. A simplified lead citrate stain for use in microscopy. *J. Cell Biol.*, **5**:163–193.

43. Vercruysse, J., Taraschewski, H. & Voigt, W. P. 1988. Main clinical and pathological signs of parasitic infections in domestic animals. *In:* Melhorn, H. (ed.), Parasitology in Focus. Springer-Verlag, Berlin, p. 486.

Received 8-26-91, 12-13-91; accepted 1-2-92

J. Protozool., 39(4), 1992, pp. 494–498

Characteristics of a Microsporidium (Protozoa: Microspora) Infecting Grasshoppers (Orthoptera: Pyrgomorphidae) in Cape Verde, Africa

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ABSTRACT. A light and electron microscopic study was conducted of a microsporidium isolated from the grasshopper *Pyrgomorpha cognata* Krauss, 1877 collected in Santo Antão and Santiago Islands, Cape Verde. The evidence suggests that although there are some differences, such as tissues affected and size of spores, the organism appears conspecific with *Nosema pyrgomorphae* Toguebaye, Seck & Marchand, 1988, which was described from another species of the genus *Pyrgomorpha* Audinet-Serville, 1838 in Senegal. However, in addition to the differences in tissue specificity and size of spores, light microscopy studies also revealed some stages of the pathogen (uninucleate bodies and plasmodia) apparently not previously observed in *N. pyrgomorphae*.

Key words. *Nosema pyrgomorphae, Pyrgomorpha.*

GRASSHOPPERS of the genus *Pyrgomorpha* Audinet-Serville, 1838 are commonly found in the Sahel Region of Africa and the Cape Verde archipelago [4]. Recently, Toguebaye et al. [14] described a microsporidium, *Nosema pyrgomorphae*, from grasshoppers of that genus in Senegal. The definitive host remains unclear because in the description the authors mentioned two different species, *P. conica* (Olivier, 1791) and *P. bispinosa* (Walker, 1870), as hosts.

During a survey for grasshopper pathogens in Cape Verde, microsporidiosis was observed in *P. cognata* Krauss, 1877 from the islands of Santo Antão and Santiago. This paper presents the characteristics of the etiological agent responsible for those infections, with particular emphasis on a comparison with the microsporidium reported by Toguebaye et al. [14].

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MATERIALS AND METHODS

Surveys for infected grasshoppers were conducted in Santo Antão, Sao Vicente, Santiago, Maio, Boa Vista, Sal, and Fogo Islands. Tissues were routinely examined for infection as fresh mounts with saline under phase contrast microscopy. Permanent preparations were air dried, fixed in methanol and stained with Giemsa as described by Wang et al. [16].

For transmission electron microscopy, tissues were fixed in 2.5% (v/v) glutaraldehyde buffered with 0.1 M cacodylate buffer, pH 7.4. Postfixation was in 1% (w/v) OsO_4 and was followed by dehydration in an ethanol series. Tissues were embedded in Spurr's resin and sections were stained with uranyl acetate followed by lead citrate and examined with a JEOL JEM CX electron microscope at 100 kV.

RESULTS

General observations. Infected grasshoppers were collected in Santo Antão and Santiago Islands. Natural prevalence of