

## Ultrastructural Study of *Endoreticulatus durforti* N. Sp., a New Microsporidian Parasite of the Intestinal Epithelium of *Artemia* (Crustacea, Anostraca)

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**ABSTRACT.** A new microsporidian parasite of the *Artemia* intestinal epithelium has been studied. The microsporidium developed within a membranous parasitophorous vesicle from the host rough endoplasmic reticulum consisting of two membranes, with the proximal one usually lacking ribosomes.

All developmental stages had isolated nuclei. Unikaryotic meronts developed into merogonial plasmodia. Merogonial division occurred by binary fission and rosette-shaped fragmentation. In young sporonts, an electron-lucent space, corresponding to the developing endospore, was immediately observed between both the plasmalemma and the exospore primordium. Sporogonial division occurred also by rosette-shaped fragmentation, resulting in at least eight sporoblasts that developed directly into spores. Fresh spores were 1.7 × 0.9 µm in size and oval-shaped. The 8-11 coil isofilar polar filament was arranged in two rows. The polaroplast was bipartite. The nature of the parasitophorous envelope, host-parasite interaction, developmental cycle and taxonomy are discussed.

**Supplementary key words.** Brine shrimp, cytology, intestinal microsporidium, ultrastructure.

**A**RTEMIA sp. is widely used in marine aquaculture as live food for fish and shrimp larvae. Microsporidiosis is common in *Artemia* populations, enhanced by high population densities [31]. Codreanu [12] first described four species of microsporidian parasites of *Artemia* (*Nosema exigua*, *Nosema artemiae*, *Plistophore myotropha*, *Gurleya dispersa*) using light microscopy. *Nosema exigua* was described as infecting the intestinal epithelium. These species have since been partially re-described by Codreanu-Balcescu & Codreanu [13] using electron microscopy. In this study *N. exigua* was interpreted as belonging to the genus *Unikaryon*. Recently a new species, *Vavraia an ostraca* Martinez, Vivarès, Medeiros, Fonseca, Andral & Bouix, 1992 [31], has been described as a muscular and intestinal parasite in an extensive culture of *Artemia* in Brazil.

The new microsporidium studied here was found in a natural parthenogenetic population of *Artemia* along the South Atlantic coast of Spain. Although, like *N. exigua*, it developed in the intestinal epithelium, all the developmental cycle, including merogony, occurred within a membranous parasitophorous vesicle, as opposed to *N. exigua*, which during merogony develops in direct contact with the host cytoplasm. Differing from *V. an ostraca*, the parasitophorous envelope was not secreted by meronts. Spore size and shape, developmental cycle, morphology and ultrastructure also revealed this new microsporidium to be different from the species already described on *Artemia*. It developed within a parasitophorous vesicle probably derived from host rough endoplasmic reticulum, closely resembling the genus *Endoreticulatus* Brooks, Becnel & Kennedy 1991 [4], and its developmental cycle was also consistent with that of *Endoreticulatus*. Therefore, it has tentatively been included in the genus *Endoreticulatus*, under the specific name of *E. durforti* n. sp.

### MATERIALS AND METHODS

The animals were collected from a solar saltern in Huelva, located on the South Atlantic coast of Spain, where the *Artemia* population is affected by chronic microsporidiosis.

The specimens were initially selected for the *N. artemiae* study (December 1989) but the intestinal microsporidiosis was also recognized. For histological and ultrastructural studies the animals were fixed in 3% glutaraldehyde buffered in 0.1 M sodium

cacodylate (pH 7.2). Fixed samples were further processed in Montpellier after 6 days of transport. Specimens fixed in glutaraldehyde were washed in sodium cacodylate buffer (pH 7.2), postfixed in 1% (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4° C and then washed again. Osmolarity of fixative, postfixative and washing solutions was adjusted to the osmolarity of the tissues, which varied according to the environmental salinity [14]. The specimens were then dehydrated in an ascending series of ethanol to absolute ethanol, transferred through an ascending series of ethanol-Spurr solutions and finally embedded in 9:1 Spurr-Epon resin. A total of 45 specimens were sectioned. Semi-thin sections were stained with toluidine blue, and ultrathin sections were stained with uranyl acetate and lead citrate.

For optical microscopy parasite characterization, digestive tracts of specimens were dissected and directly observed for fresh spores. Then they were smeared in frotis, fixed with methanol, and stained using the Giemsa technique. Permanent preparations were mounted in DePeX. All measurements were made using a micrometer ocular at 1,000×.

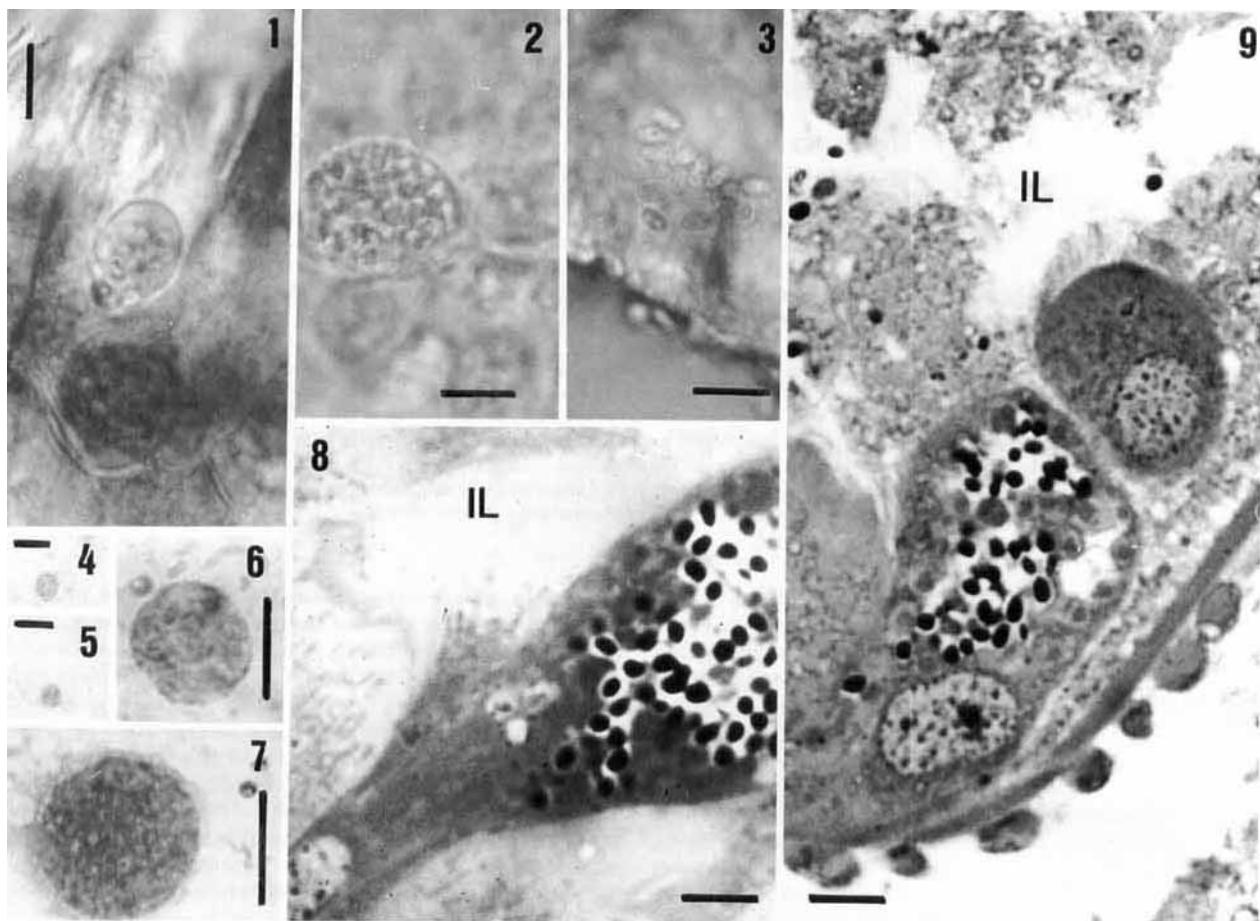
### RESULTS

From the 45 animals selected for the study of microsporidiosis on *Artemia*, 23 were parasitized by *N. artemiae* and 12 showed the intestinal microsporidium. Only eight of the specimens parasitized by *N. artemiae* were also affected by the intestinal microsporidiosis.

**Optical microscopy.** The microsporidium studied herein infected the intestinal epithelium cells. In fresh digestive tracts a parasitophorous vesicle could be distinguished in some epithelial cells (Fig. 1, 2) projecting into the intestinal lumen. In semi-thin sections of the digestive tract the parasites were usually located at the anterior pole of the cells (Fig. 8, 9). Parasitized cells were progressively hypertrophied and parasites were finally ejected into the intestinal lumen (Fig. 9). The host nucleus did not appear to be hypertrophic.

In smears, supposed meronts were distinguished as round isolated cells with one or two nuclei (Fig. 4, 5) and measuring 2 µm in diameter. Unfortunately merogonial division forms or young sporonts were not distinguished. Next developmental stages observed were included in subpersistent vesicles. Presumed sporoblasts and immature spores were observed in number up to 128 per vesicle (Fig. 6, 7), which measured up to 19 µm in diameter.

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**Fig. 1-9.** Optical microscopy of *Endoreticulatus durforti* n. sp. **1.** Parasitophorous vesicle emerging from the intestinal epithelium. Bar = 6.25  $\mu\text{m}$ . **2.** Live intestinal epithelium cell containing a parasitophorous vesicle. Bar = 6.6  $\mu\text{m}$ . **3.** Immature live spores. Bar = 6.25  $\mu\text{m}$ . **4, 5.** Presumed meronts in smears. Bar = 3.25  $\mu\text{m}$ . **6.** Parasitophorous vesicle containing supposed sporoblasts in smears. Bar = 5.7  $\mu\text{m}$ . **7.** Parasitophorous vesicle containing spores in smears. Bar = 13  $\mu\text{m}$ . **8.** Parasite projection into the intestinal lumen included in a portion of the host cell. Semithin section. IL: intestinal lumen. Bar = 5.5  $\mu\text{m}$ . **9.** Hypertrophy of a parasitized intestinal cell, in semithin section. Free spores can be observed in the intestinal lumen. IL: Intestinal lumen. Bar = 5.5  $\mu\text{m}$ .

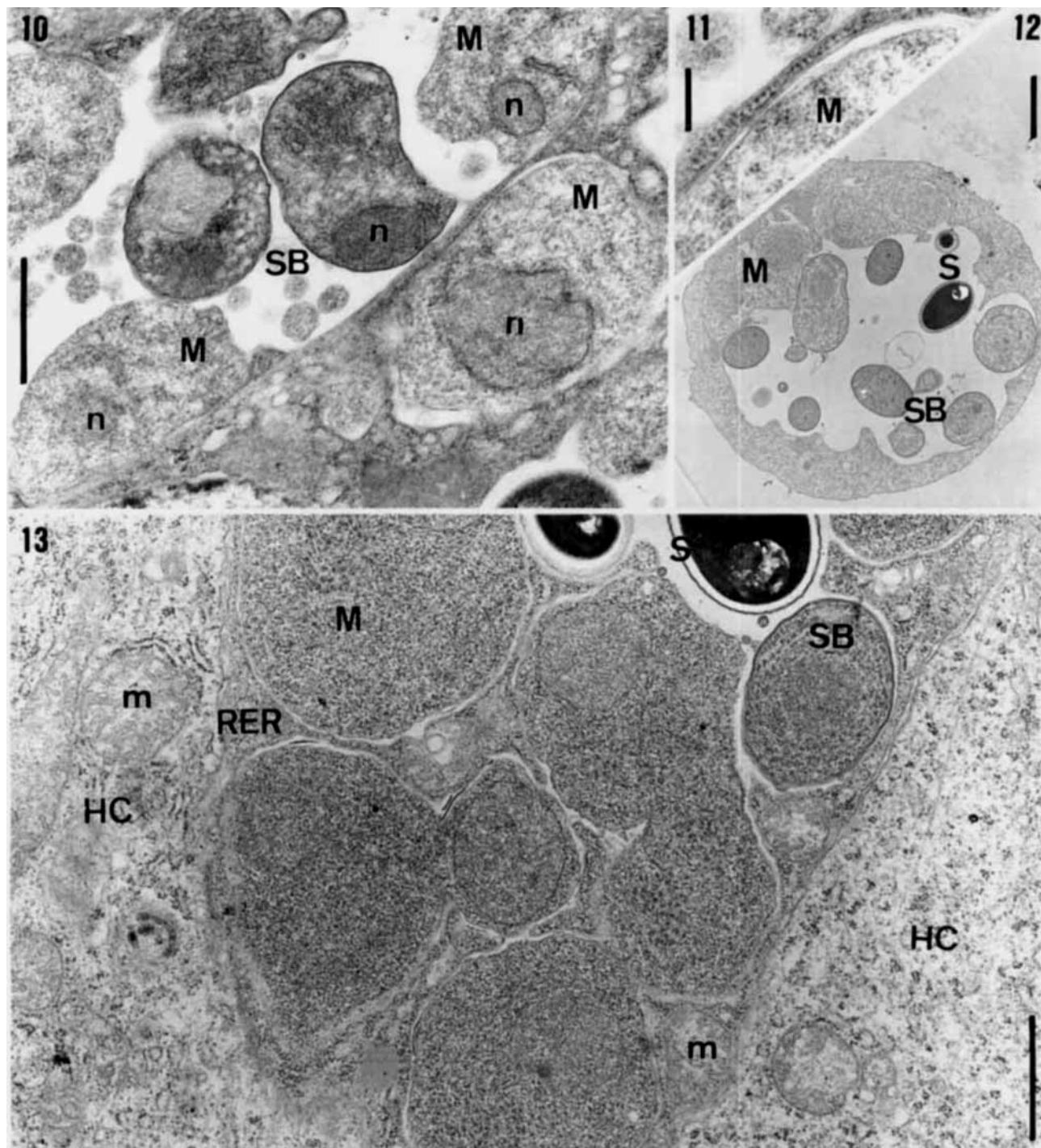
**Ultrathin observations of parasitophorous vesicle.** Ultrastructural studies confirmed all developmental stages developed within parasitophorous vesicles. The parasitophorous envelope, a double membrane, was composed of an outer ribosome-studded membrane, and an inner membrane lacking ribosomes (Fig. 10, 11). It seemed to be from the cisternae of host rough endoplasmic reticulum. The inner membrane was closely associated with the meront plasmalemma, while the outer membrane was occasionally continuous with rough endoplasmic reticulum from host cell cytoplasm. No secretory activity in the meront plasmalemma was observed. Moreover, the spatial dimensions between the inner and outer membranes were similar to those of host rough endoplasmic reticulum. Apparently different parasite stages within a unique parasitophorous vesicle could be seen (Fig. 10), but vesicle fusioning or fragmentation could happen. Pieces of host cytoplasm could be observed between different parasite stages lying in an apparently single vesicle (Fig. 10, 12). By the other side, different parasitophorous vesicles within a unique host cell could be seen, host cytoplasm being imbricated between parasites and speaking for the close association of host metabolism and parasite development (Fig. 13).

**Pre-sporal stages.** The earliest stage observed in ultrathin sections was round unicellular meronts occluded by the membranous envelope of the parasitophorous vesicle (Fig. 14). Al-

ways delimited by a unit membrane 7 nm thick, young meronts showed a uniform cytoplasm rich in dispersed ribosomes while mature meronts presented a more electron-dense cytoplasm. A number of ribosomes became associated with membranes to the endoplasmic reticulum, which was successively arranged around the nucleus (Fig. 19). Merogonial plasmodia with two to four nuclei were observed in section (Fig. 15, 16, 19).

Ultrathin sections study revealed merogonial division to occur by binary fission in early merogony (Fig. 15, 19). Nevertheless at the end of merogony more electron-dense plasmodia, surrounded only by a unit membrane, divided by rosette-shape fragmentation (Fig. 17, 18). During the plasmodia fragmentation the parasitophorous vesicle could follow the fragmentation line, the merogonial division products being separated by thin portions of host cytoplasm bound within the double membrane of host rough endoplasmic reticulum (Fig. 18). The number of merogonial generations is unknown.

The onset of the sporogony, classically recognized by the presence of the spore wall primordium, was here noticeable when sporont plasmalemma was clearly separated from the membrane of the parasitophorous vesicle (Fig. 20). Sometimes, a mielinic-like band was placed between the parasitophorous envelope and the parasite plasmalemma (Fig. 16) but no secretory activity on the plasmalemma was observed, differing from the



**Fig. 10-13.** Ultrathin pathology. **10.** Ultrathin section showing sporoblasts (SB) lying free in the matrix of the parasitophorous vesicle, while meronts (M) are closely associated to the parasitophorous envelope. Probably fusion of different parasitophorous vesicles has occurred. n, nucleus. Bar = 1.3  $\mu$ m. **11.** Detail of the nature of the parasitophorous envelope, consisting of a double membrane. The inner one lacks ribosomes, but the outer is continuous with host rough endoplasmic reticulum. M, meront. Bar = 260 nm. **12.** Infected portion of an exposed host cell in the intestinal lumen. M, meront; SB, sporoblasts; S, spore. Bar = 200 nm. **13.** Close association of host rough endoplasmic reticulum and mitochondria with parasites, M, meront; SB, sporoblast; HC, host cytoplasm; m, mitochondrion; RER, rough endoplasmic reticulum. Bar = 540 nm.

discontinuous layer of electron-dense material secreted by the sporont plasmalemma which correspond to the spore wall primordium. The spore wall secretion was observed to begin in division forms (Fig. 21). The outer layer progressively thickened and the discontinuous segments of electron-dense material fused

(Fig. 21-23). The exospore primordium was almost immediately separated from the membrane by an electron-lucent space, corresponding to the primordium of the endospore. The well-developed sporogonial wall was 25 nm thick.

Sporogonial division occurred before the sporogonial wall was

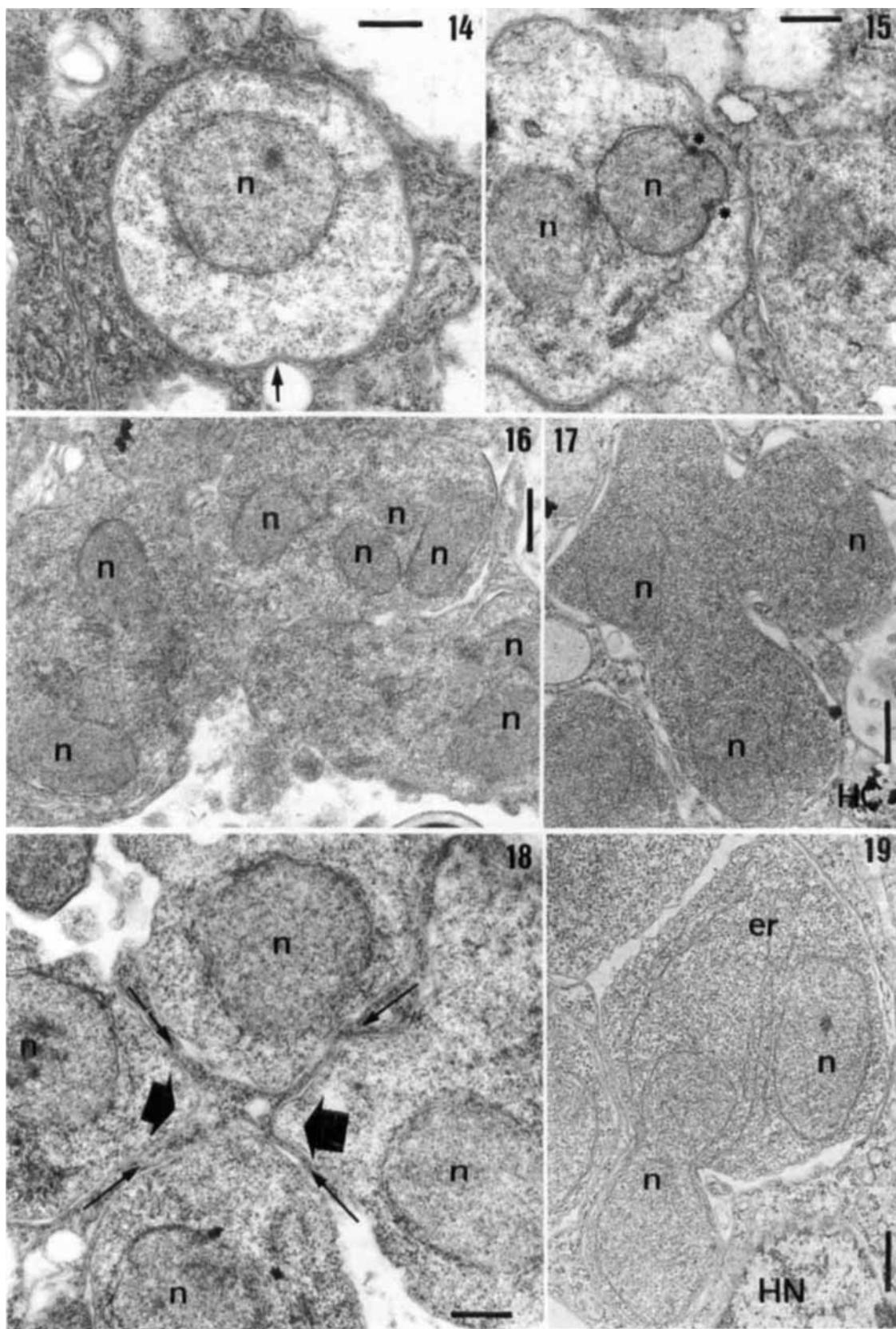


Fig. 14-19. Merogony of *E. durforti*. 14. Young meront occluded within a double membrane-bound vesicle (arrow). Bar = 250 nm. 15. Young meront fragmentation. Meront with two nuclei still in division. Spindle plaques can be distinguished. n, nucleus. Bar = 600 nm. 16. Mature merongial plasmodia with 2 and 4 nuclei in section. n, nucleus. Bar = 410 nm. 17. Mature merongial plasmodium dividing by rosette-shape

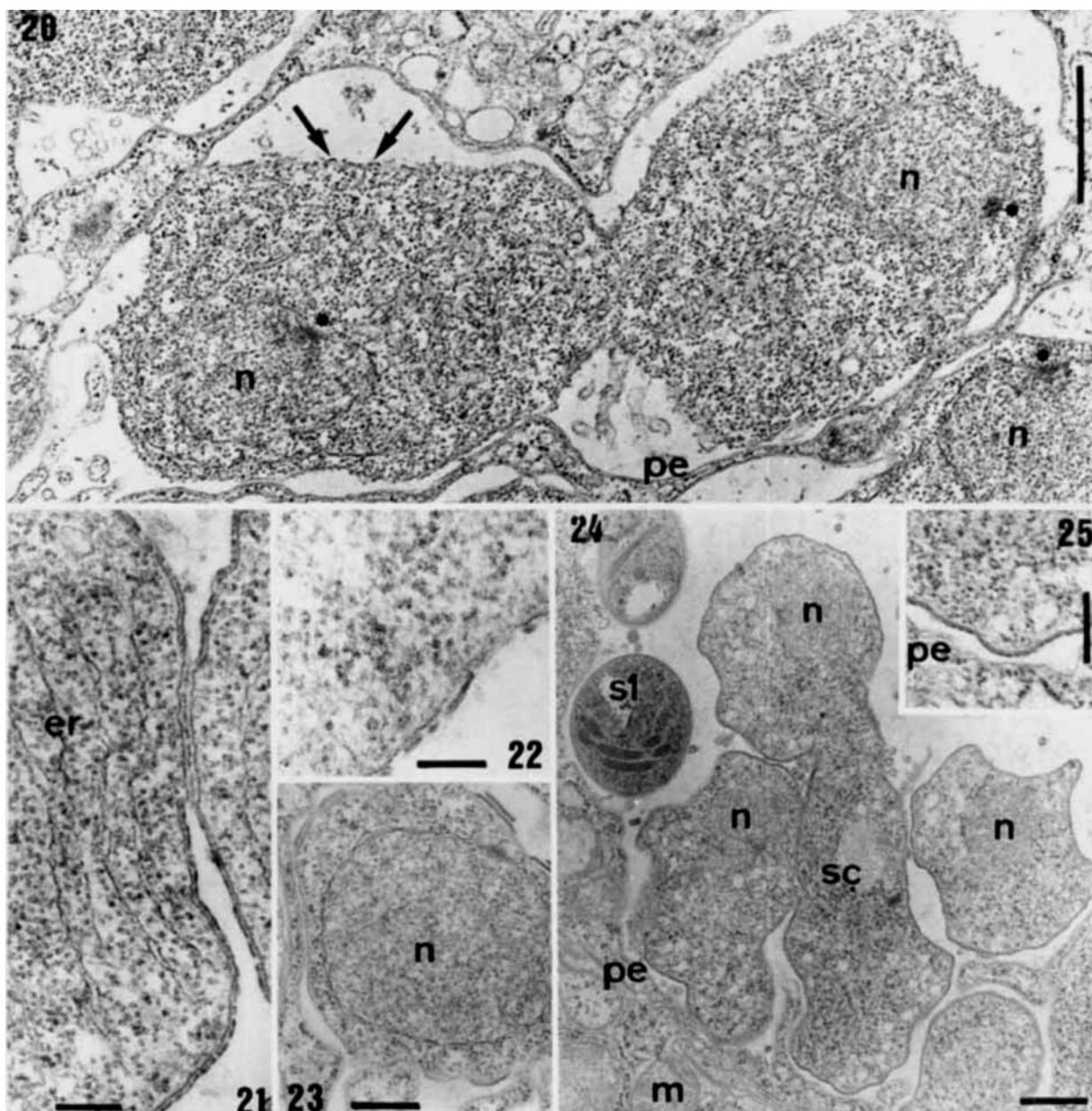


Fig. 20–25. Sporogony of *E. durforti*. 20. Early deposit of electron-dense material on the plasmalemma (arrows). Parasites become more and more separated from the parasitophorous envelope (pe). Spindle plaques (asterisks) can be observed in nuclei. Bar = 1.1  $\mu$ m. 21, 22. Discontinuous electron-dense layer in sporont. The primordium of the endospore can be observed as an electron-lucent space between the discontinuous exospore and the plasmalemma. er, endoplasmic reticulum. Bars = 187 nm (21); 125 nm (22). 23. Laminar secretions of electron-dense material associated with exospore generation. n, nucleus. Bar = 330 nm. 24. Sporogonial division by rosette-shaped fragmentation occurring before the sporogonial wall becomes continuous. m, mitochondrion; n, nucleus; pe, parasitophorous envelope; S1, immature spores; sc, scindosomes (= paramural bodies). Bar = 625 nm. 25. Detail of the discontinuous sporogonial wall in the sporogonial plasmodium. pe, parasitophorous envelope. Bar = 285 nm.

fragmentation. No evidence of secretory activity in the plasmalemma can be noticed. n, nucleus. Bar = 660 nm. 18. Merogonial uninucleated product after fragmentation of the merogonial plasmodium. Host rough endoplasmic reticulum (thin arrows), closely follows the fragmentation line and surrounds the division products (thick arrows). n, nucleus. Bar = 275 nm. 19. Merogonial plasmodium in supposed binary division process. Ribosomes became associated with endoplasmic reticulum. HN, host nuclei; n, nucleus; er, endoplasmic reticulum. Bar = 330 nm.

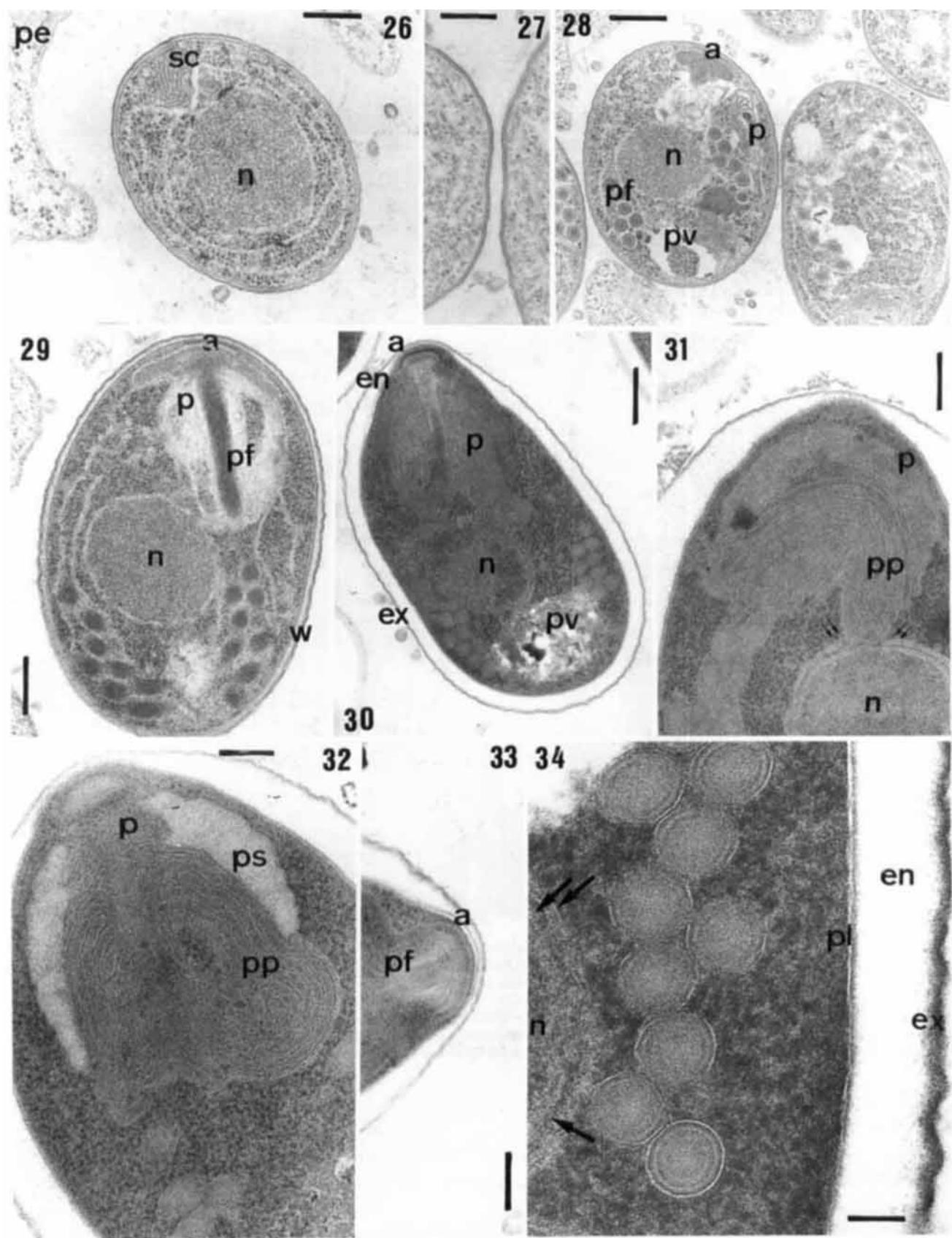


Fig. 26-34. Sporogenesis and spore ultrastructure of *E. durforti*. 26. Sporoblasts, showing a continuous wall. Scindosomes can still be observed, while the anchoring disc is already distinguished. n, nucleus; pe, parasitophorous envelope; sc, scindosomes (= paramural bodies). Bar = 285

absolutely continuous (Fig. 24, 25). Fragmentation into uninucleated products (sporoblasts) occurred, as in last merogony, by simultaneous fission and rosette-shaped structures were usually observed. Synaptonemal complexes or other signs of reductional division were not observed. Neither the sporogonial plasmodium nor the newly formed sporoblasts were covered with a continuous exospore layer (Fig. 26–28). The separation between the sporogonial plasmodia and the parasitophorous envelope increased while sporoblasts and spores remained in the center of the vesicle. Sporogenesis occurred with no further nuclear division and followed the general pattern for microsporidia. The sporoblast wall was equivalent to that of the sporogonial plasmodium. This wall was only continuous in mature sporoblasts that showed the primordia of the spore organelles (Fig. 29).

**Spores.** In fresh condition spores were oval-shaped. Immature fresh spores measured  $2.1\text{--}2.5 \times 0.9\text{--}1.5 \mu\text{m}$ , in front to  $1.7 + 0.15 \times 0.9 + 0.12 \mu\text{m}$  ( $n = 25$ ) for mature spores (Fig. 3). In ultrathin longitudinal sections, the unikaryotic spores showed the typical cytology of microsporidian spores, with an anteriorly bipartite polaroplast, a polar filament, a central and isolated nucleus, and a posterior vacuole (Fig. 30, 31). The nucleus was surrounded by an additional double membrane, probably derived from the endoplasmic reticulum cisternae (Fig. 31, 34, arrows). At the anterior pole, the bipartite lamellar polaroplast was organized around the rectilinear part of the polar filament, with the membranous saccules being 7 nm thick anteriorly and 21 nm thick at the posterior part. The anterior polaroplast was only composed of a few lamellae. The anchoring apparatus (Fig. 33), polar sac and anchoring disc, was typically umbrella-shaped, with the anchoring disc measuring up to 300 nm in diameter. The polar sac was largely expanded and covered most of the polaroplast (Fig. 32).

The 8–11-coil polar filament was arranged in two rows (Fig. 30, 34). The filament was isofilar, with the section measuring 97 nm thick. Internally the filament was composed of alternating layers of different electron densities. Externally, a unit membrane of approx 5 nm surrounded the filament. The trilayered spore wall was of constant thickness in different spores, measuring 140–150 nm. It was typically composed of an external 30-nm-thick exospore, an intermediate electron-lucent endospore and an inner 7-nm unit membrane. No external ornamentation or difference in the electron density of the exospore was observed (Fig. 34).

## DISCUSSION

The presence of microsporidia in the intestinal epithelium of *Artemia* has been known since 1957. Codreanu [12] described a new species, *Nosema exigua*, based only on optical microscopy observations, but after a later ultrastructural study [13] it was identified as *Unikaryon exiguum*. In contrast to the microsporidium studied herein, *U. exiguum* develops within a parasitophorous vesicle only during sporogony, merogonial stages lying in direct contact with host cytoplasm. Moreover, polysporous

development is not observed in *U. exiguum*, which always divides by binary fission. *Vavraia anostraca* can also infect the intestinal epithelium of *Artemia* sp., but it develops within sporophorous vesicles secreted by the meront plasmalemma [31].

**Polymorphism.** The simultaneous presence of both microsporidia, *N. artemiae* and the intestinal microsporidium treated herein, in a specimen led us to think about the possibility of this microsporidiosis being caused by a unique but polymorphic microsporidium. Microsporidia with complex cycles have been described [1, 2, 16, 17, 18, 38], alternating sequences of development corresponding to the two microsporidia-type patterns. Usually, this kind of microsporidium is affected by reductional division, and two types of spores can be observed. In *Spraguea lophii* [29] a *Nosema*-type cycle alternating with *Nosemoides*-type development resembles the presence of *Nosema* and the intestinal microsporidium in *Artemia*. Nevertheless, some elements led us to consider the individuality of both microsporidia.

On the one hand, synaptonemal complexes have never been found either in *Nosema artemiae* or in the intestinal microsporidium. On the other hand, the intestinal microsporidiosis seems to be limited to the intestinal epithelium, with parasites being ejected only into the intestinal lumen. Moreover, the stage connecting both microsporidia (diplokaryotic meronts in the microsporidium studied here or unikaryotic meronts in *Nosema artemiae*) has never been observed. High percentages of animals affected by both microsporidiosis would suggest the possibility of a polymorphic microsporidium being responsible for both tissue parasitisms. But, the totality of specimens studied were almost entirely sectioned and only 35% of the specimens being parasitized by *N. artemiae* showed the other microsporidium.

Microsporidia are sometimes not polymorphic in a unique host. Some microsporidia require an obligate intermediate host to be infective in the target host. *Amblyospora* sp. [38], *Amblyospora connecticus* Andreadis, 1990 [1], and *Parathelohania anophelis* Kudo, 1924 [2] have a copepod as an intermediate and obligate host to parasitize mosquitoes. The saltern from which the infected biomass studied here originated is located in an area of salt marshes where mosquito populations are very abundant. Hence the possibility of this infection being caused by a microsporidium also parasitizing mosquitoes must be evaluated. Probably the only way to corroborate this hypothesis would be through experimental infections. Meanwhile, we tentatively propose the individuality of those microsporidia.

**Host-parasite interaction.** A particularly interesting aspect of the pathology of the microsporidium studied here is the close association between the host cytoplasm and the parasitophorous vesicle, especially in merogonial stages. It is widely known that microsporidia lack mitochondria [11] and that they are present in tissues where they easily gain energy from the host cells [17, 40, 42, 48]. The proximity between these cytoplasmic host organelles and the parasites supports this hypothesis, as well as the probable existence of some kind of energy transport-chain between hosts and parasites. An additional participation of host

nm. 27. Detail of the wall of mature sporoblasts. Bar = 175 nm. 28. Mature sporoblasts showing the primordia of spore organelles. a, anchoring disc; n, nucleus; p, polaroplast; pf, polar filament; pv, posterior vacuole. Bar = 400 nm. 29. Mature sporoblast, with polaroplast in development around the rectilinear part of the polar filament. a, anchorage disc; n, nucleus; pf, polar filament; w, wall. Bar = 300 nm. 30. Longitudinal section of a mature spore. a, anchorage disc; en, endospore; ex, exospore; n, nucleus; p, polaroplast; pv, posterior vacuole. Bar = 250 nm. 31. Mature spore showing the nucleus surrounded by two double membranes, the outer one being connected with the posterior polaroplast (arrows). n, nucleus; pp, posterior polaroplast. Bar = 140 nm. 32. Anterior part of a mature spore. Notice the largely expanded polar sac that surrounds almost entirely the polaroplast. p, anterior polaroplast; pp, posterior part of the polaroplast; ps, polar sac. Bar = 153 nm. 33. Anchoring disc. a, anchoring disc; pf, polar filament. Bar = 160 nm. 34. Transverse section of the coiled polar filament, showing the internal organization. A double system of membranes surrounds the nucleus (arrows). en, endospore; ex, exospore; n, nucleus; pl, plasmalemma. Bar = 70 nm.

to parasite development can occur in the microsporidium treated herein, through the close association between host endoplasmic reticulum and this microsporidium, as already proposed by several authors [35, 50]. The cisternae lacuna surrounding the parasites show the same electron density as the rest of the host rough endoplasmic reticulum, thus the synthetic performance is probably active. Ralphs & Matthews [35] discussed the advantages of the relationship between host endoplasmic reticulum and the merogonial stages of *Microgemma* included within endoplasmic reticulum vesicles. They proposed that the proliferative form of the parasite placed in the host cell synthetic system has a ready supply of materials, and is directly related to the host cell nucleus, thus constituting a link of possible major significance in host cell control. In support of this hypothesis, Vivarès, Richard & Ceccaldi [44] observed a high complementarity between free amino acids found in different genera of microsporidia and its crustacean hosts (marine decapods), thus covering the spectrum of both host and parasite essential amino acids. In this sense, the special characteristics of the rRNA of microsporidia, closely associated to that of prokaryotes [15, 46], is worth noting. This is also an evidence of the extremely ancient origin of microsporidia [36, 47]. However, further physiological and cytochemical studies are required.

**Parasitophorous envelope.** In microsporidian infections of the gut epithelium, spores enclosed within the plasma membrane of the host cell have frequently been observed when microsporidia-filled cells are shed into the gut lumen [23]. This has erroneously suggested the presence of sporophorous vesicles. In the generic assignment of microsporidia that undergo multisporous sporogony within a vesicle, it is important to know whether the vesicle is of parasite origin and thus a sporophorous vesicle, or of host origin and thus a parasitophorous vesicle. New genera have been created based on this characteristic [10].

In the microsporidium studied herein the envelope of the vesicle including parasites seems to be closely related with host rough endoplasmic reticulum, being, therefore, a parasitophorous vesicle. The outer membrane, with associated ribosomes, is frequently in continuity with host rough endoplasmic reticulum. The inner membrane seems to be the proximal side of the cisternae of host rough endoplasmic reticulum from which ribosomes are lost. Similarity in the spatial dimensions of the lacuna between the inner and outer membranes and those of the rough endoplasmic reticulum cisternae also supports this hypothesis. Usually, due to the weakness of the 5-nm-thick membranous parasitophorous envelope, the parasites are liberated within the intestinal lumen. In this way, the fusion and rupture of these membranous parasitophorous vesicles is, apparently, very frequent and it can erroneously suggest the idea that different developmental stages of the parasite are included within a unique vesicle.

Numerous microsporidia developing within a membranous parasitophorous vesicle have been previously described. *Nolleria pulicis* Beard, Butler & Becnel, 1990 [3], *Cytopsporogenes fidelis* Canning, Barker, Nicholas & Page, 1985 [10], *Baculea daphniae* Loubès & Akbarieh [29], *Tetramicra brevifilum* Matthews & Matthews, 1980 [32], *Pleistophora* sp. Percy, Wilson & Burke, 1982 [34] and *Encephalitozoon cuniculi* (Levaditi, Nicholau & Schoen, 1923) [37] develop within single membrane-bound vesicles. Cisternae of host endoplasmic reticulum have been described around meronts and/or other stages of *U. exiguum* [13], *Glugea anomala* Moniez, 1887 [8], *Nosema wistmani* Canning, Wigley & Barker, 1983 [10], *Pleistophora* sp. [34], *Microgemma hepaticus* Ralphs & Matthews, 1986 [35], *Microsporidium novacastrensis* Jones & Selman, 1985 [19], *M. itiiti* Malone, 1985 [30], *Endoreticulatus fidelis* Brooks, Becnel

& Kennedy, 1988 [4] and *E. shubergi* (Zwölfler, 1927) Cali & El Garhy [6].

The presence of different developmental stages within a unique parasitophorous vesicle has been reported by Sprague & Vernick [37] for *Encephalitozoon cuniculi*, and by Percy et al. [34] for *Pleistophora* sp., but it is probably due to the disintegration of the vesicle or to the asynchronous development of meronts and sporonts. *Encephalitozoon cuniculi* parasitizing macrophages [49] are enclosed within a parasitophorous vesicle that is considered to be the phagocytic vacuole. Canning et al. [10] argue that the occasional presence of different developmental stages within a vesicle can be determined by an eventual division and fusion of the parasitophorous vesicles.

Contrary to the importance given by many authors to the origin and nature of the envelope-bounding parasites, the development within a parasitophorous vesicle of host origin has also been interpreted as an attempt to isolate the parasites [24], and it seems to have lost its taxonomical significance [21, 25]. Weiser [51] established that xenoparasitic complexes in invertebrates are syncytial xenomas, with host cells confluent in one common syncytium, or neoplastic xenomas, in which the parasite induces an increase in the number of host cells inside the lobe of infected tissue. Based on the pathology we have studied, we propose that the parasitophorous vesicle constructed from the host rough endoplasmic reticulum is another characteristic of the host-parasite interaction, frequently observed in infections of the intestinal epithelium [3, 4, 19, 28, 30, 34].

**Developmental cycle.** Rosette-budding occurring both in merogony and sporogony (even if binary fission seems to be also frequent during merogony) is quite exceptional in the described microsporidia development. These characteristics, as well as the polysporous development within a parasitophorous vesicle and the nuclear condition showing isolated nuclei during the whole life cycle, are the main distinctive features of the developmental cycle of the microsporidium treated herein.

Sometimes it is difficult to identify a microsporidium because descriptions are usually based on the study of heavily infected specimens, and vegetative stages are frequently absent. The nuclear condition and the merogonial division were frequently supposed or extrapolated, not directly observed [23]. Moreover, the absence of merogony has sometimes been suggested [22]. *Cystosporogenes* [10] and *Nosemoides* Vinckier, 1975 [41], for example, have been described with no direct observations of merogonial division. In the micrograph reported by Loubès & Akbarieh [27] for *N. simocephali*, a parasite of the intestinal epithelium of the Cladocera *Simocephalus vetulus*, the onset of sporogony can be observed to occur in daughter plasmodia derived by plasmotomy from a merogonial plasmodium. This observation is of additional interest because the description of *Nosemoides simocephali* confirmed the genus *Nosemoides*.

The onset of sporogony is considered to be marked by the secretion of the spore wall electron-dense material through the sporont plasmalemma. In microsporidia with a polysporous development within parasitophorous vesicles the early shrinkage of meronts from the parasitophorous envelope usually occurs at the same time. Nevertheless, in the microsporidium studied here we only observe the secretory activity when this separation is already important, and moreover, usually occurring in division forms before the last merogonial fragmentation has finished. Also differing from the usual sequence in microsporidia development, endospore generation does occur almost concurrently with exospore generation. Usually, the exospore is secreted by the sporont and the endospore begins developing in sporoblasts [38]. However, early generation of the endospore, as we have seen, has sometimes been observed. In *Nudisporea biformis* Larsson [25] described the almost simultaneous gen-

eration of both layers of the spore wall in sporonts. This phenomenon can also be distinguished in the micrographs reported by Desportes [17] for *Stempellia mutabilis* Léger & Hesse, 1910, and by Voronin [45] for *Lanatospora macrocyclospis*, as discussed by Larsson for *Agglomerata* [26]. Other divergences from the established pathway of microsporidia development have been described, notably for the genus *Enterocytozoon* Desportes, Charpentier, Galian, Bernard, Cochon-Priollet, Lavergne, Ravisse & Modigliani, 1985 [5]. Therefore, the microsporidia development pattern must not be considered as being a fixed process, but rather adapted to the relative physiology of each parasite and its interactions with the host.

Contrary to the meiotic division observed at the onset of sporogony in certain species [16, 18, 20, 31, 24, 43], the absence of synaptonemal complexes suggests that the microsporidium we studied has an asexual life cycle. Sporogonial division by rosette-shaped fragmentation of plasmodia is a widespread developmental characteristic of microsporidia. In the Pleistophoridae family, the *Vavraia* species divide by plasmotomy and rosette-budding during the sporogony [7], as is also the case for the Thelohanidae, Tuzetidae, and Telomyxidae families and the *Nosemoides*, *Cougotella*, *Endoreticulatus*, *Tetramicra* genera [23]. Sporogenesis in this case follows the normal pathway in most microsporidia, with no further nuclear division.

**Taxonomical assignment.** According to the classification guide established by Larsson [23] for the identification of microsporidian genera, the following genera are polysporous and have unpaired nuclei during the entire life cycle: *Cystosporogenes*, *Duboscqia*, *Glugea*, *Loma*, *Microgemma*, *Nosemoides*, *Orthosoma*, *Pleistophora*, *Spraguea*, *Tuzetia* and *Vavraia*. The newly established genus *Flabelliforma* Canning, Killick-Kendrick & Killick-Kendrick, 1991 [9] has also been described as having these characteristics. Always, according to Larsson's key, for the polysporous genera with unpaired nuclei, *Microgemma*, *Nosemoides*, and *Tetramicra* show sporogonial plasmotomy in rosette-budding but do not develop within a sporophorous vesicle. Only in the species *Hessea squamosa* Ormières & Sprague, 1973 [33], merogonial division by rosette-like fragmentation has been observed in diplokaryotic merogonial plasmodia, but in addition to the differences in the nuclear condition, typical merogony occurs only by binary fission. The following described genus *Endoreticulatus* [4] deserves special mention. This microsporidium has isolated nuclei, is a parasite of the intestinal epithelium and shows a polysporous development within a parasitophorous vesicle that is very similar to the parasitophorous vesicle observed by us. The vesicle of *Endoreticulatus* has been recognized as being functionally equivalent to a sporophorous vesicle, being relatively subsistent [4] and thus including only one developmental stage of the parasite. The merogonial and sporogonial division modalities (binary and/or multiple fission) are adequate for the microsporidium here studied, although typical ribbon-like meronts have not been observed in the *Artemia* intestinal microsporidium.

The polysporous development within vesicles greatly resembles the *Pleistophora*-complex [6, 8], which has a total of nine different genera. The microsporidium we study clearly differs from the genus *Pleistophora* for the envelope to be originated from host rough endoplasmic reticulum, contrary to the parasite origin in the *Pleistophora* genus. Following the key to the genera of the *Pleistophora*-complex elaborated by Canning et al. [9], between the genera showing unpaired nuclei during the whole life cycle, only *Endoreticulatus* has a parasitophorous envelope similar to that of the microsporidium studied by us. The other characteristics used in this key are also consistent with our observations. This genus presumably includes unikaryotic polysporous microsporidia developing within a parasitophorous

vesicle derived from host rough endoplasmic reticulum. In this sense, recently a new combination has been created by Cali & El Garhy 1991 [6] for the ancient *Pleistophora schubergi* Zwölfer, 1927, now redescribed and identified as *Endoreticulatus schubergi* (Zwölfer, 1927) n. comb.

Although the characteristics of genus *Endoreticulatus* can agree with the microsporidium here discussed, the creation of a new species is, under our point of view, largely justified. Ribbon-like meronts have not been observed as in *E. fidelis*. Typical rosette-shape fragmentation of sporogonial plasmodia here described has not been illustrated for *E. fidelis*. Spore size greatly differs ( $2.26 \times 1.51 \mu\text{m}$  for *E. fidelis*;  $1.7 \times 0.98 \mu\text{m}$  for the microsporidium here studied), and the polar filament is differently arranged (two rows in front of the one row for *E. fidelis*), the number of coils being also clearly different (8–11 coils for the microsporidium treated by us; 6–7 coils for *E. fidelis*). The number of spores by vesicle can be up to 128 while in *E. fidelis* it is around 30. Moreover, the almost simultaneous secretion of the exospore and endospore could be considered of taxonomical interest and it is not described to happen in the type species of the genus *Endoreticulatus*. Furthermore, ecologically *E. fidelis* parasitizes a terrestrial insect while the microsporidium we describe parasitizes an aquatic crustacean. In summary, we have tentatively identified the microsporidium here studied to belong to the genus *Endoreticulatus*, naming it *E. durforti* n. sp.

## TAXONOMY SUMMARY

*Endoreticulatus durforti* n. sp.

Meronts, round, measure 2  $\mu\text{m}$  in diameter. Fresh spores, oval, are  $1.7 \times 0.98 \mu\text{m}$ . Polar filament (97 nm in section) arranged in two rows. 8–11 coils. Parasitophorous vesicles containing over 128 spores, measuring up to 19  $\mu\text{m}$  in diameter.

**Histopathology.** Intestinal epithelium.

**Type host.** *Artemia* sp. (Crustacea, Anostraca).

**Type locality.** Huelva (South-Atlantic coast of Spain).

**Etymology.** The specific name comes from our colleague, Prof. Mercedes Durfort.

**Deposition of types.** The holotype, accession number MNHN-P93.0, is in the Protozoa Collection, Museum National d'Histoire Naturelle, Paris, France. Paratypes in the author's collection.

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