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Microsporidiosis on *Artemia* (Crustacea, Anostraca): light and electron microscopy of *Vavraia anostraca* sp. nov. (Microsporidia, Pleistophoridae) in the Brazilian solar salterns

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ABSTRACT

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A high prevalence of microsporidiosis was detected in an extensive culture of *Artemia* in two Brazilian solar salterns. The disease was responsible for the disruption of both the eggs and the biomass production. The main microsporidian is primarily a muscular parasite, with pansporoblastic development. All stages presented isolated nuclei. In merogonial stages, the amorphous coat divided with the cell, whereas in the sporogonial plasmodia the protoplasm retracted away from the coat, producing the vesicule cavity. Sporogonial division occurred both stepwise and by rosette-shape fragmentation. The number of nuclei in mature sporonts was variable and sometimes exceeded 64. As the sporophorous vesicle became mature, the matrix was occupied by metabolic granular products that became tubular. Microspores ($3.3 \times 2.1\ \mu m$) and macrospores ($4.1 \times 2.7\ \mu m$) were observed. The number of coils of the anisofilar filament was 11 (microspores) and 15 (macrospores). The ultrastructure and cytology of the parasite revealed that it was a new species: *Vavraia anostraca* sp. nov. For the first time a species of *Vavraia* is described in crustaceans. The taxonomy is discussed.

INTRODUCTION

Since its inoculation in 1977, the presence of "white spots" has been observed in *Artemia* in two solar salterns in N.E. Brazil affecting less than 2% of

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the total population. An increase of this phenomenon was noticed in 1989, when microsporidiosis affected the whole adult population, especially during dry periods. No infection in early stages was reported. Although these "white spots" were mainly located in the abdomen (Fig. 1), they were also present in the last thoracic segments. The infection was normally associated with a suboptimal nutritional state and a biomass density higher than 100 ind. l^{-1}.

Microsporidiosis in *Artemia* was first reported by Codreanu (1957) who described massive microsporidiosis in the saline lake of Tékirghiol (Rumania) caused by four species: *Nosema exigua, Gurleya dispersa, Pleistophora myotropha* and *Glugea artemiae,* later transferred by Sprague (1977) to the genus *Nosema.* Nevertheless, microsporidiosis in *Artemia* was not longer studied until a massive and chronic microsporidiosis caused by *Nosema artemiae* was recently detected on the South Atlantic coast of Spain (Martinez, 1989; Martinez et al., 1989).

The morphological and ultrastructural study of the main microsporidian has revealed important differences with all the microsporidia already described in *Artemia.* Moreover, these results have induced us to propose it as a new species: *Vavraia anostraca* sp. nov.

MATERIAL AND METHODS

The animals were collected from Macau in Northern Brazil, and primarily fixed in 5% formaldehyde. Further samples were fixed in 3% glutaraldehyde buffered in 0.1 *M* sodium cacodylate (pH 7.2). The specimens were washed in 0.2 *M* sodium cacodylate buffer (pH 7.2), postfixed in 1% (*w/v*) osmium tetroxide in cacodylate buffer and washed again. The specimens were embedded in 90% Spurr–10% Epon resin. Ultrathin sections were stained with uranyl acetate and lead citrate. For optical microscopy the sections were stained with toluidine blue. Smears of fixed material (5% formaldehyde) were stained with eosin and methylene blue.

RESULTS

Optical microscopy

Semithin sections revealed the presence of close-packed bundles of spores invading the muscular tissue. The parasite was also commonly found infecting the haemocele and the intestinal epithelium (Figs. 2 and 3). The youngest stages observed were round uni-binucleated cells (Fig. 4), which were assumed to be young meronts. By karyokinesis they developed into plasmodia with isolated nuclei surrounded by an amorphous coat. Dividing by plasmotomy, the number of nuclei and the thickness of the external coat increased during merogonial maturation. Sporogonial stages were recognized developing within the sporophorous vesicle. Cytoplasmic fission, by gradual frag-

mentation into smaller and smaller fragments, resulted in uninucleated sporoblasts (Fig. 7). Immature sporophorous vesicles included an undetermined number of uninucleated sporoblasts, which developed directly into spores (Fig. 8).

In fixed and stained smears, the vesicles containing mature spores were between 11 and 21 μm in diameter (mean value 16.6; $n=25$), (Fig. 6) according to the number of spores. The fixed and stained spores were oval shaped and of various sizes (Fig. 5). The most frequent macrospores (10:1) measured 4.1×2.7 μm ($n=25$) and the size range was $3.5–5.0\times2.0–3.0$ μm. The microspores were 3.3×2.1 μm ($n=25$) and the size range was $2.8–3.5\times1.5–2.0$ μm, the former corresponding to the largest sporophorous vesicle. The number of spores per vesicle was variable, only the largest spores being packed in numbers of 16 and exceptionally 8. The most common grouping for both types of spores was 64.

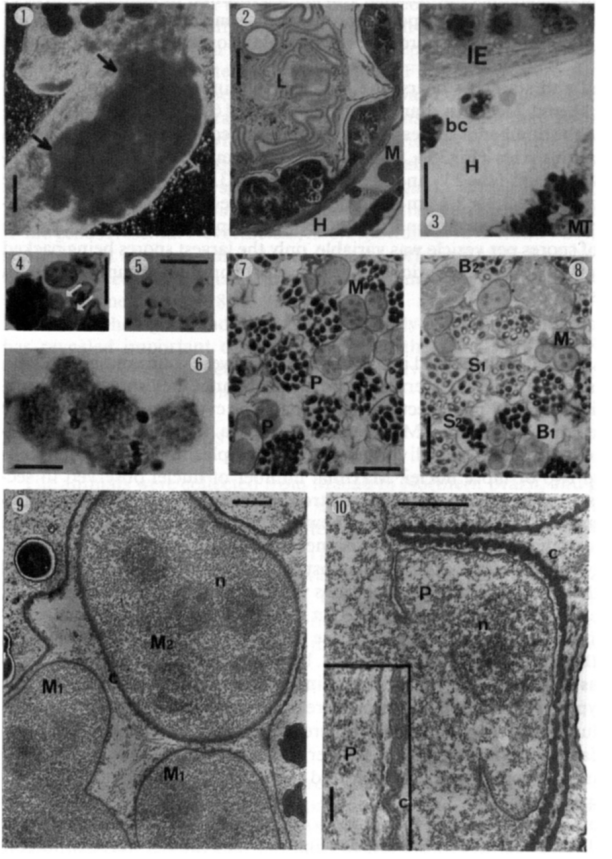
Electron microscopy

The earliest stages observed in ultrathin sections were young plasmodia with isolated nuclei (Fig. 9). External to the plasmalemma they showed an irregularly thick (53–143 nm) electron-dense coat. The cytoplasm was rich in free ribosomes and polysomes. Merogonial plasmodia fragmented by plasmotomy, the amorphous coat dividing with the cytoplasm. The daughter cells contained two or more nuclei. Maximal number of nuclei observed in sections was 11. The thickness of the electron-dense coat increased as maturation progressed. Mature plasmodia showed a less electron-dense cytoplasm due to the partial arrangement of ribosomes into rough endoplasmic reticulum.

In early sporogony the external coat lost contact with the plasmalemma and became the envelope of the sporophorous vesicle (Fig. 10). This 125-nm thick envelope, highly folded, was organized at this stage in two electron-dense layers separated by an electrolucent space (Fig. 10 inset). Simultaneously a gradual thickening of the plasmalemma, by deposition of electron-dense material, was observed. The vesicle matrix included granular dispersed material.

Two types of sporogonial division were observed within the membrane:
(a) gradual fragmentation into cytomeres followed by division into uninucleated daughters via smaller and smaller fragments (Fig. 11)
(b) simultaneous division, uninucleated sporoblasts being isolated from rosette shapes (Fig. 12).

The episporontal space of the sporophorous vesicles containing sporoblasts temporally showed tubular inclusions (Fig. 13). The tubules were cylindrical, approximately 53 nm wide in section, being absent from the mature spore-containing vesicles. The young sporoblasts showed an apparent Golgi apparatus from which both the polar sac and the polar filament derived. Mature sporoblasts, with an almost impermeable exospore, presented an opaque cytoplasm where only the coils of the polar filament were evident.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The completely developed sporophorous wall presented a bilayered structure, having lost the folded character of previous stages (Fig. 15). The spore ultrastructure (Fig. 14) was similar for both microspores and macrospores, differing only in numerical characters. The spore wall was 94–97 nm thick, with 7-nm-thick plasmalemma; an electrolucent endospore; and a 35-nm-thick electron-dense exospore with a more electron-dense surface coat (Fig. 18). At the anterior half of the spore, the polaroplast was structured in an apical part (P1) with tightly packed lamellae (5.4–10.8 nm wide), and a posterior region (P2) with more expanded lamellae (11.0–16.2 nm wide). But there was no gap between the two parts (Fig. 16).

The anisofilar polar filament was arranged in one or two layers (Fig. 17). The microspores had 8–9 wide and 3 thinner coils arranged in one layer. The macrospores had 10–12 wide and 5–6 narrow coils arranged in two layers. The wider filament coils measured 90 nm in microspores and 120 nm in macrospores, and the thinner ones 70 nm in microspores and 92 nm in macrospores. The rectilinear part of the filament was anteriorly attached to the anchorage apparatus that showed the typical umbrella structure. The anchoring disc measured up to 325 nm in diameter, and the polar sac was prolonged laterally, covering the anterior polaroplast lamellae (Fig. 16).

The transversely sectioned polar filament revealed an internal organization in concentric layers having different electron densities. It was covered externally by a 7-nm-thick unit membrane (Fig. 19).

Fig. 1. Manifestation of microsporidiosis in an adult female. The big "white spot" (arrows) appears dark because the photography was done on a black field. (Bar: 200 μm).

Fig. 2. Transverse section of a parasitized intestinal epithelium. L, intestinal lumen; H, haemocele; M, merogonial plasmodium. (Bar: 20 μm).

Fig. 3. Transverse section showing both muscular (MT) and intestinal epithelium (IE) infected tissues. bc, blood cells; H, haemocele. (Bar: 10 μm).

Fig. 4. Young meronts (arrows) between mature spores and a mature merogonial plasmodium. (Bar: 10 μm).

Fig. 5. Fixed and stained spores in smears. (Bar: 10 μm).

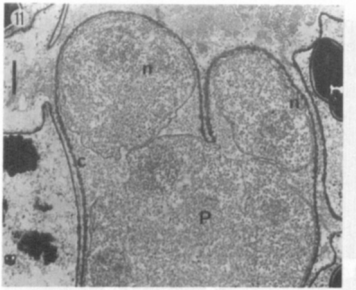
Fig. 6. Fixed and stained sporophorous vesicles containing spores in smears. (Bar: 10 μm).

Fig. 7. Final merogony and stepwise sporogonial division in semithin section. M, mature merogonial plasmodia; P, sporogonial plasmodium in fragmentation. (Bar: 10 μm).

Fig. 8. Uninucleated sporoblast maturing into spores. B1, young sporoblasts; B2, mature sporoblasts; M, merogonial plasmodium; S1, immature spores; S2, mature spores. (Bar: 10 μm).

Fig. 9. Ultrathin section of merogonial plasmodia surrounded by an amorphous coat. c, amorphous coat; M1, young merogonial plasmodium; M2, mature merogonial plasmodium; n, nucleus. (Bar: 1 μm).

Fig. 10. Early sporogony, the protoplasm retracting away from the amorphous coat. c, amorphous coat; n, nucleus; P, sporogonial plasmodium. (Bar: 700 nm). Inset: Detail of both deposition of electron-dense material on the plasmalemma surface, and the highly folded electron-dense bilayered amorphous coat. (Bar: 125 nm).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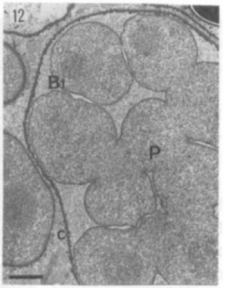
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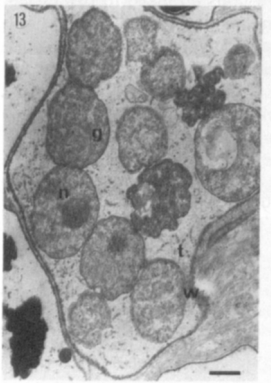
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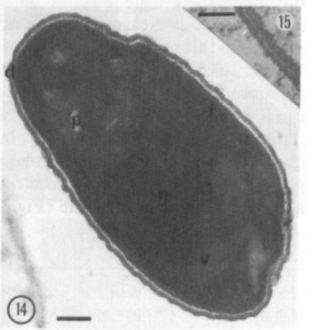
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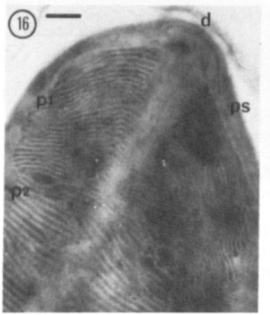
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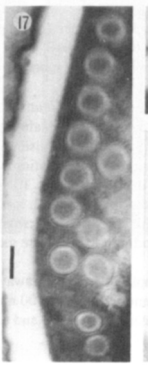
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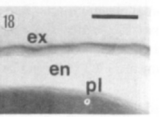
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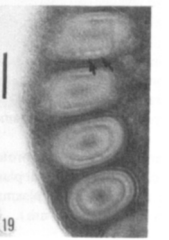
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DISCUSSION

Microsporidia can be considered as one of the most pathogenic and infectious protozoan parasites in crustaceans (Sprague, 1977; Vivares and Azevedo, 1988); they belong to various genera.

In the Brazilian *Artemia* microsporidiosis, life cycle (polysporous sporogony developing within a sporophorous vesicle) and parasite cytology revealed the major organism responsible for the infection to belong to the *Pleistophora* group. The parasite developed in direct contact with host tissues without xenoma-formation as is usual in fishes (Faye et al., 1990). But the comparison of the biometrics and pathological data with those reported by Codreanu for *Pleistophora myotropha* revealed important differences. Additionally, Codreanu observed the microspores to be dominant and grouped in tetrasporous vesicles, but in the Brazilian *Artemia* they were less abundant (1:10) and usually grouped in a number higher than 32. Moreover, vesicles with 8–16 spores were in this case very rare, 64 being the most frequent grouping, and up 128. We must also take into account that both *Artemia* populations differed greatly in biology and biogeography (Lake Tékirghiol — athalasohaline inland European lake with a parthenogenetic population; Brazil — coastal solar salterns with a bisexual strain of *Artemia*).

Since the genus *Pleistophora* was redescribed (Canning and Nicholas, 1980), the taxonomic position of most *Pleistophora* from invertebrates has been questioned and ultrastructural redescriptions are needed. The main characters to differentiate the genera of the polysporous microsporidia developing within a sporophorous vesicle are based on ultrastructural studies. With re-

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Fig. 11. Sporogonial plasmodium (P) dividing by stepwise fragmentation. c, amorphous coat; n, nucleus. (Bar: 1 μm).

Fig. 12. Sporogonial plasmodium (P) dividing by rosette-shaped fragmentation. c, amorphous coat; Bl, young sporoblasts. (Bar: 1 μm).

Fig. 13. Sporophorous vesicle containing young sporoblasts. g, Golgi apparatus; n, nucleus, t, tubular inclusions; w, sporophorous wall. (Bar: 1 μm).

Fig. 14. Immature spore. d, anchorage disc; f, polar filament; n, nucleus; p, polaroplast; v, vesicle-like cytoplasmic inclusions. (Bar: 250 nm).

Fig. 15. Completely developed sporophorous wall showing a smooth electron-dense bilayered structure. (Bar: 100 nm).

Fig. 16. Polaroplast and anchorage apparatus. d, anchorage disc; ps, polar sac; p1, anterior polaroplast; p2, posterior polaroplast. (Bar: 150 nm).

Fig. 17. Transverse section of the coils of the anisofilar polar filament in a mature spore. (Bar: 100 nm).

Fig. 18. Trilayered spore wall in a mature spore. ex, exospore; en, endospore; pl, plasmalemma. (Bar: 100 nm).

Fig. 19. Transverse section of the wide coils of the polar filament. Arrows show the electron-lucent spots embedded in a more electron-dense layer, typical in other *Vavraia* species (Bar: 100 nm).

spect to the nature and origin of the sporophorous envelope, as well as the nuclear condition, Canning and Hazard (1982) distinguished three genera in *Pleistophora* Gurley 1893: namely *Pleistophora, Vavraia* and *Polydispyrenia*. This latter clearly differed from the former *Pleistophora typicalis* Gurley 1893, by the presence of diplokarya in vegetative stages and the reductional division in sporogony. *Pleistophora* and *Vavraia* are considered to be very close and both are assigned to the family Pleistophoridae. They differ basically in the manner of sporogonial plasmodium fission and the presence of channels in the amorphous coat during merogony. While in *Pleistophora* the uninucleated sporoblasts come from stepwise division (developing occasionally in macrospores) and the amorphous coat is permeated by channels, in *Vavraia* the sporogonial plasmodium undergoes multiple fission by rosette formation into uninucleated sporoblasts, excluding the formation of macrospores (Canning and Hazard, 1982). Larsson (1986) supported the deletion of the statement on rosette-like division in the sporogony of *Vavraia*, and added the possibility of formation of a smaller number of macrospores and the anisofilar character of the filament contrary to the isofilar filament of *Pleistophora*. Further, it seems to be assumed that *Pleistophora* may be found in vertebrates and *Vavraia* in invertebrate hosts.

From these considerations and in accordance with the ultrastructure of the microsporidian studied here, we can conclude that the polysporous microsporidian parasite of this *Artemia* population belongs to the genus *Vavraia*, closely resembling the type species of this genus, *Vavraia culicis* Weiser 1947 (Canning and Hazard, 1982). For the first time a *Vavraia* species is described in crustaceans.

DESCRIPTION

Vavraia anostraca sp. nov.

Merogony: Merogonial plasmodia with isolated nuclei surrounded by an external electron-dense highly-folded coat. Division by plasmotomy, the external coat accompanying the protoplasm.

Sporogony: Sporogonial plasmodia with a thickened plasmalemma retract away from the external coat and divide by either stepwise or rosette-shaped division, producing both macrospores and microspores. Octosporous sporophorous vesicles with macrospores are rare, 64 and 32 vesicles being most frequent for both spore types. Groups of 16 and up 128 spores can be found.

Spores: Uninucleate, oval and with a large posterior vacuole. Dimensions (fixed and stained): macrospores, 3.5–5.0 × 2.0–3.0 μm; microspores, 2.8–3.5 × 1.5–2.0 μm. Spore wall 94–97 nm thick, with a 35-nm electron-dense exospore, externally more electron-dense.

Sporophorous vesicle: Spherical. The sporophorous wall is divided into two

electron-dense layers by a translucent space. Tubular inclusions in the matrix of vesicles containing sporoblasts.

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

Histopathology: Parasitic in muscular tissue, haemocele and intestinal epithelium of *Artemia*.

Type locality: Macau, N.E. Brazil.

Etymology: Named after the Order Anostraca.

Deposition of types: Author's collection.

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