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Ultrastructure of *Tricornia muhezae* N. G., N. Sp. (Microspora, Thelohaniidae), a Parasite of *Mansonia africana* (Diptera: Culicidae) from Tanzania

JUDITH K. PELL[1,2] and ELIZABETH U. CANNING

Department of Biology Imperial College of Science, Technology and Medicine, Prince Consort Road, London, SW7 2AZ, United Kingdom

ABSTRACT. Collections of *Mansonia africana* mosquito larvae were made at one site in N.E. Tanzania in 1985 and 1987 and from two additional sites, both within about 2 mi of the original one in 1987. An octosporous microsporidian, present at all three sites, was found in both years infecting from 7 to 22% of larvae. Spores (stained in Giemsa) measured 3.0 μm ± 0.25 μm × 2.25 μm ± 0.26 μm. Ultrastructurally, spores were seen to have an anterior rim surrounding a depressed area where the endospore was at its thinnest. In transmission electron microscopy section, the rim appeared as two processes into which all layers of the wall extended. At the posterior end all layers of the wall extended into a simple knob-like structure which could be interpreted as a section through a crest running longitudinally around the spore. The polar filament was anisofilar, with two anterior coils of greater diameter than the three posterior coils. Although most closely resembling the genera *Amblyspora* and *Parathelohania* in the family Thelohaniidae, the species in *M. africana* differs from the former, which has oval spores, broadly rounded at the ends, and from the latter, which has a prominent, ridged posterior extension to the spores. The new species has been placed in a new genus and the name *Tricornia muhezae* proposed.

Key words. Culicine mosquito, microsporidium, taxonomy.

MOSQUITOES (Diptera, Culicidae) are hosts to numerous species of microsporidia, the life cycles of which are extremely varied. Some life cycles, initiated on ingestion of spores by larvae, are completed in a single host generation. According to genus these parasites may have isolated or paired (diplokaryotic) nuclei throughout the life cycle, e.g. *Vavraia culicis* (isolated nuclei) and *Nosema algerae* (diplokarya).

The more complex life cycles involve at least two generations of the host [4, 5, 10, 12]. In the first larval generation, gametogenesis and cytoplasmic fusion of gametes (plasmogamy) have been reported [6], giving rise to diplokaryotic stages culminating in diplokaryotic spores in adult females. Sporoplasms ejected from these spores infect the ova so that infections are carried over to the second larval generation. In these larvae two types of sporogony ensue, one involving meiosis and producing (usually eight) meiospores (e.g. *Microsporidium aedis, Culicosporella lunata, Amblyospora* spp. [3, 6, 11, 24]) and the other, not involving meiosis, producing binucleate spores (*Culicosporella lunata*) or uninucleate spores (*Microsporidium aedis, Culicospora magna* [7]), responsible for horizontal transmission to new hosts.

The life cycle of *Amblyospora dyxenoides* from *Culex annulirostris* is still more complex, requiring a copepod intermediate host *Mesocyclops albicans* [23, 24]. This alternation of hosts has also been found for *Amblyospora connecticus* in *Aedes cantator* and *Acanthocyclops vernalis* [2, 3] and has been suggested for *Amblyospora californica* in *Culex tarsalis* and an un-named copepod [6]. Thus, the asexual phase giving rise to pyriform uninucleate spores directly infective to mosquito larvae takes place in a copepod. The remaining stages responsible for transovarial transmission and meiospore production take place in the two generations of mosquito. Meiospores are infective to copepods.

In 1985 an octosporous microsporidian species was found in *Mansonia africana* mosquitoes from Tanzania. Previously *Amblyospora* spp. have been reported from *Mansonia dyari* and *Mansonia leberi* [12], but no information on locality or structure was given. In this paper the development of the species from *M. africana* is described and its taxonomic position discussed.

MATERIALS AND METHODS

Mansonia africana is a common mosquito found in ponds and swamps throughout the tropics. The larvae respire by attaching their siphons to the roots of floating vegetation [17]. Using the techniques of Service [21], several collections were made in 1985 and 1987 from the same site, a small pond of about 4 m in diameter at the village of Kisiwani near Muheza, N.E. Tanzania (5° 11′ S, 38° 5′ E). On the second occasion larvae were also obtained from another small pond (Mamakingi Pond near Tengeni, Tanzania) and from a swamp (Muheza Sisal Estate swamp near Muheza, Tanzania), both about two miles from the first site.

All larvae that could be dislodged from a sample of water lettuce plants were collected to determine the proportion infected at each of the three sites. Overtly infected larvae, recognisable by their swollen and opaque white thoracic and abdominal segments, were used as experimental material. Infected individuals with no overt symptoms were diagnosed by microscopy.

For light microscopy, smears of larvae were air dried, fixed in methanol and stained for 30 min in 10% Giemsa stain (Gurr's improved R66), buffered at pH 7.2. Small pieces of infected mosquitoes were also fixed on site for transmission electron microscopy. The siphon was removed for specific identification and the body cut into 1-mm lengths, which were transferred to Karnovsky's fixative for 10 min at ambient temperature and then into fresh fixative for 1 h at 4° C. After washing twice in 0.12-M sodium cacodylate buffer, the material was not processed further until return to the United Kingdom laboratory 1–3 mo later. It was then post-fixed in 2.5% osmium tetroxide, block-stained in phosphotungstic acid and uranyl acetate, dehydrated in acetone and embedded in Spurr's resin. Gold sections were cut on a Reichert ultramicrotome, further stained in alcoholic uranyl acetate and lead citrate, and viewed on a Phillips 300 transmission electron microscope.

Spores liberated from larvae and kept at 4° C in water containing antibiotics were purified on a 50% Percoll gradient [15] and photographed and measured in a monolayer on agar.

RESULTS

Early infections were found in cells of the fat body, but after multiplication soon filled the entire larval body. The mean incidence of infection from collections at the original site (Kisiwani), including those larvae with infections but no overt signs,

[1] To whom correspondence should be addressed.

[2] Current address: Crop & Environment Protection Division, Entomology & Nematology Department, Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ, United Kingdom.

was 17.74% in 1985 and 13.2% in 1987. At the other two sites where collections were made in 1987, the average incidence was 21.5% at Mamakingi Pond and 6.9% at Muheza Sisal Estate Swamp.

Light microscopic appearance. Measurements of fresh octospores from the 1985 and 1987 collections were 3.10 μm ± 0.12 μm × 2.14 μm ± 0.12 (n = 13) and 3.20 μm ± 0.20 μm × 2.36 μm ± 0.34 μm (n = 13), respectively. They were broadly ellipsoid in shape, with one end flattened (Fig. 1, 2). Giant spores were occasionally seen (Fig. 3) and were presumed to result from incomplete division during sporogony.

In Giemsa-stained smears (1985 collection) the earliest stages seen were diplokaryotic and thought to be late meronts or early sporonts (Fig. 4). After separation of the two nuclei of the diplokaryon and two nuclear divisions, tetranucleate and finally octonucleate sporonts were produced (Fig. 5), which gave rise typically to eight sporoblasts (Fig. 6), and then matured into spores (Fig. 5). Sometimes the host cell nucleus and/or large accumulations of metabolic granules were present at the periphery of the sporont (Fig. 5, arrows). These gave the impression of supernumerary parasite nuclei but could be distinguished by their differential staining characteristics. The terminal flattening of the octospores was also visible after staining (Fig. 6, arrow), but was not a feature of giant spores (Fig. 7). Stained spores measured 3.0 μm ± 0.25 μm × 2.25 μm ± 0.26 μm (n = 22).

Ultrastructural appearance. Although elution of tissues, probably resulting from the long period in buffer, gave less than ideal fixation, the principal ultrastructural features of the parasite were preserved. Meronts were rounded cells with rather empty cytoplasm traversed by endoplasmic reticulum, studded with ribosomes and limited by a thin unit membrane. The large nuclei were in diplokaryotic arrangement, often with condensed chromatin and occasionally showing electron-dense patches on the nuclear envelope that were probably spindle plaques, signifying a past or incipient karyokinesis (Fig. 8).

At the onset of sporogony the two nuclei of the diplokaryon drew apart (Fig. 9). An envelope, later to become the sporophorous vesicle, was laid down outside the plasma membrane. As the envelope separated from the plasma membrane, clumps of electron-dense material were deposited between the two, some of which became distributed over the surface of the sporont, ultimately forming a complete electron-dense coat (Fig. 9, arrows).

Further development of the sporont involved nuclear division and separation of the cytoplasm into eight uninucleate sporoblasts. Lobed sporonts representing incompletely separated sporoblasts, and irregularly shaped stages just after separation were seen (Fig. 10). Although sections rarely revealed more than three or four buds of a dividing sporont in one plane, the presence of octonucleate plasmoidia in Giemsa-stained smears confirmed that division was typically delayed until the octonucleate stage. Premature division of the sporont before completion of the final nuclear division could result in formation of giant sporoblasts and spores as seen by light microscopy. Subsequent to division, the sporoblasts first acquired a regularly oval shape (Fig. 11). At a later stage before maturing into spores, sporoblasts appeared crenated (Fig. 12) probably because of poor penetration of fixative.

Within the sporophorous vesicles, accumulations of metabolic products gave differing appearances as the spores matured. In the younger vesicles they were predominantly in large aggregates which were electron dense with lighter patches (Fig. 10). Other types were tubular or pale homogenous spheres (Fig. 11, 12). As maturation proceeded, the tubular and homogeneous elements increased and the large aggregates became less electron dense. In vesicles containing mature spores there was little evidence of any of the metabolic products, although some structures resembling concentric lamellae persisted. Occasionally a structure, resembling a host cell nucleus, was observed within the boundary of the now degenerate host cell but outside the sporophorous vesicle (Fig. 11).

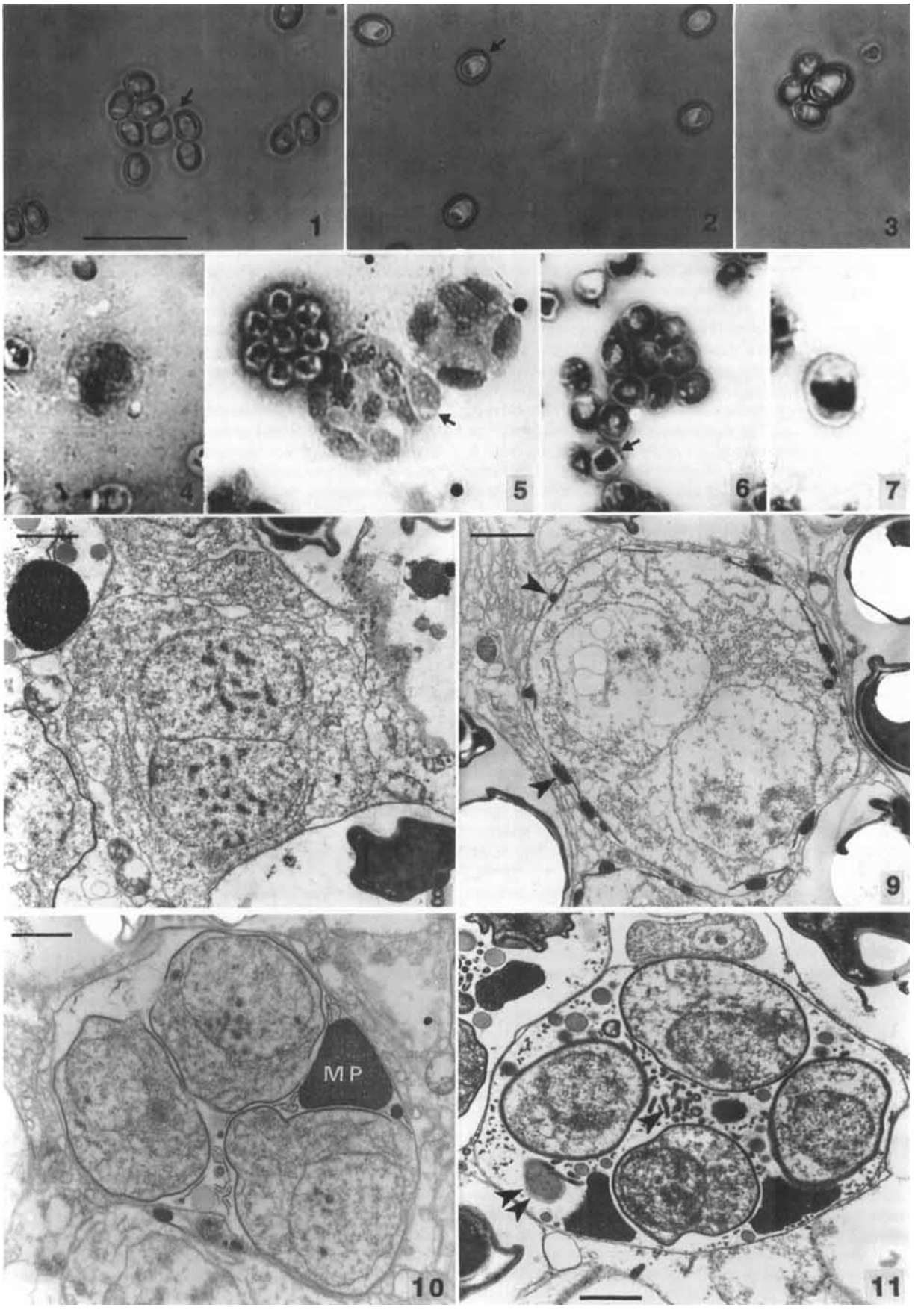
The surface coat of the developing sporonts and sporoblasts increased in thickness and several layers could be distinguished at the stage of crenation (Fig. 13). The endospore was first laid down as a mottled layer of moderate electron density, but it finally resolved as an inner electron lucent layer and an outer layer of moderate electron density within the fine electron-dense exospore (Fig. 14, 15). The total thickness of the wall along the sides of the spore was 0.17 μm.

Internally, the mature spore had an anterior polar cap extending outward as fine processes beneath the endospore. The polar filament was anisofilar, with two anterior coils of greater diameter than the three posterior coils. The basal region of the filament was surrounded by compact layers of polaroplast lamellae (Fig. 15). The remaining area of the spore was occupied by a single nucleus and general cytoplasm containing cisternae of endoplasmic reticulum studded with ribosomes. The latter were particularly clear in slightly immature spores (Fig. 14).

At the anterior end of the spore corresponding to the flattened end seen by light microscopy, longitudinal sections showed the spore wall was raised into two processes into which all layers of the wall extended (Fig. 15). The constant appearance of these structures as knobs, whatever the angle of sectioning, is consistent with the structure of an anterior rim surrounding a depression in the region of the polar cap. At the posterior end there was a single knob-like projection, also bounded by all layers of the wall. In all transverse sections (31 sections through all regions of the spore) two lateral processes were present, suggesting a crest/ridge running longitudinally around the spore (Fig. 16, spore on the right hand side) and explaining the knob-like projection seen in longitudinal section (Fig. 15). This projection was not detected by light microscopy suggesting that its size in transmission electron microscopy may have been exaggerated by the fixation procedure. Its consistent appearance in all longitudinal sections, however, strongly implies that it is not an artefact.

DISCUSSION

The family Thelohaniidae comprises a number of genera having sporonts that undergo three nuclear divisions to give rise to eight uninucleate microspores enclosed in a sporophorous vesicle. Of the genera in the family, the species described here from *M. africana* has closest affinities with *Thelohania, Amblyospora* and *Parathelohania.* According to current criteria, the genus *Thelohania* is inadequately defined since the type species (*T. giardi* Henneguy, 1892, a parasite of the shrimp *Crangon vulgaris*) was described photomicroscopically at a time when the finer ultrastructural differences separating the octosporous genera were unknown [13]. For this reason, some species attributed to the genus *Thelohania* may be better placed in *Amblyospora, Parathelohania* or other genera. Johnson et al. [14] noted the early descriptions of *T. giardi* [13, 19] in which sporogony was reported as a series of three binary fissions. They observed identical sporogony in *Thelohania butleri* from the shrimps (*Pandalus jordani*) and suggested that this might be an important diagnostic character of the genus. If an octonucleate sporogonial plasmodium is not produced in the genus *Thelohania,* the octosporous species from *M. africana* cannot be ascribed to it. In addition, the coils of the polar filament in the genus *Thelohania*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are isofilar [12]. In the species from *M. africana* the coils are anisofilar.

Amblyospora and *Parathelohania* are dimorphic in their mosquito hosts, one sporogonial sequence giving rise in adult females to free binucleate spores which are responsible for transovarial transmission to the next generation of larvae, and the other, through octosporous sporogony, giving rise to eight meiospores in a sporophorous vesicle. The type species were designated by Hazard & Oldacre [12], as *A. californica* Kellen & Lipa, 1960 and *P. legeri* Hesse, 1904. Species known up to 1977 are listed by Sprague [22]. Since then, further mostly unnamed species have been described or recorded [1, 8, 16, 18, 20, 25–27]. Few papers describe the sequence resulting in isolated binucleate spores in adult females [4, 5, 9]. Recently Sweeney et al. [23, 24] and Andreadis [2, 3] have described, in two *Amblyospora* species, a further developmental sequence in an alternate copepod host showing this genus to be polymorphic. It is not known whether *Parathelohania* also requires an alternate host to complete its life cycle although this seems likely because octospores produced in second generation mosquito larvae are not directly infective to other larvae [10].

Although diplokaryotic meronts were found in benign infections in *M. africana* larvae, it has not been possible to link these directly with diplokaryotic stages, which are presumed to occur in adult females. In adult females such stages are the putative precursors of the octosporous development in the next generation of larvae. When eggs from wild caught adult *M. africana* that were subsequently shown to be infected with diplokaryotic parasites were reared to larvae, none were infected with the octosporous species but a high proportion of each batch turned out to be infected with a multisporous microsporidian species (this will be described in a separate publication).

Amblyospora and *Parathelohania* are distinguished by the structure of the octospores. Octospores of *Amblyospora* are oval, broadly rounded at their ends but appearing truncate, barrel-shaped, when fixed. Those of *Parathelohania* are constricted and ridged at the posterior end where the lucent endospore layer is greatly thickened. At the anterior end the endospore is thinned so that the polar cap is only just separated from the exospore and the wall is raised as a rim which appears as two ridges in section. When viewed by phase contrast microscopy there is a characteristic internal bottle-shaped appearance.

The octospores of the microsporidium from *M. africana* differ from those of *Amblyospora,* which have a simple oval or truncated outline. They resemble the octospores of *Parathelohania* in that they have a distinct anterior rim involving all layers of the spore wall and a posterior constriction. However, the posterior constriction is in the form of a simple knob derived from a lateral ridge not visible by light microscopy, in contrast to the extensively ridged and constricted region, visible by light and transmission electron microscopy in *Parathelohania.*

Clearly the parasite of *M. africana* is closer to *Parathelohania* than to *Amblyospora.* However, if we assign the species to *Parathelohania* it would be necessary to modify the generic diagnosis of that genus to allow for the reduced posterior extension of the spore wall. This seems undesirable in view of the very consistent posterior development in the numerous species of *Parathelohania* already described.

For this reason, and because *Parathelohania* is typically a parasite of anopheline mosquitoes, the parasite of *M. africana* is placed in a new genus and species, for which the name *Tricornia muhezae* is proposed, the generic name referring to the three processes (horns) consistently seen in longitudinal sections of the spore and the specific name referring to the type locality.

Tricornia n. g.

Meronts are diplokaryotic stages bounded by unthickened plasma membranes. Sporogony is octosporoblastic and is initiated when the two nuclei of the diplokaryon separate and a sporophorous vesicle is laid down external to the plasma membrane. Electron-dense metabolic products are secreted into the sporphorous vesicle cavity and some of this material is deposited on the plasma membrane to form an electron-dense coat (the future exospore). The remainder accumulates as electron-dense masses with more lucent stippling and later disperses as tubular elements and pale homogeneous spheres which almost disappear in vesicles containing mature spores. After two nuclear divisions the octonucleate sporongonial plasmodium divides into uninucleate sporoblasts which mature into uninucleate spores.

The sporophorous vesicle is fine and membrane-like. The spore wall is raised up as a rim around an anterior depressed area where the endospore layer is thinnest. In longitudinal section there is a knob-like projection at the posterior end and in transverse section two lateral processes. These observations suggest a longitudinal crest running around the spore. The polar filament is anisofilar.

The generic name derives from the three projections (horns) seen in longitudinal section.

Type material is deposited at the Natural History Museum, Zoology Department, Cromwell Road, London, SW7 5BD, U.K. (accession number 1991:4:10:1).

Tricornia muhezae n. sp. (type species)

Spores measure 3.2 ± 0.2 μm × 2.6 ± 0.3 μm. The polar filament has two anterior coils of greater diameter than the posterior three coils.

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Fig. 1–11. Photomicrographs and transmission electron micrographs of *Tricornia muhezae* from *M. africana* larvae. **1.** Purified fresh spores from the 1985 collection. Note flattened end of spores (arrow). **2.** Purified fresh spores from the 1987 collection. Note flattened end of spores (arrow). **3.** Purified fresh giant spore. **4.** Late meront or early sporont showing diplokaryotic arrangement of nuclei. **5.** Octospores and two sporonts. Apparently supernumerary sporont nuclei (arrow) with different staining properties may be a host cell nucleus or an aggregate of metabolic products in the sporophorous vesicle. **6.** Eight sporoblasts within a sporophorous vesicle. Also, sporoblasts and spores released from ruptured vesicles. Some spores showing flattened pole (arrow). **7.** Giant spore. **8.** Diplokaryotic meront. Note thin unit membrane, condensed chromatin within the nucleus and electron-dense patches on the nuclear envelope, possibly representing centriolar plaques. **9.** Early sporont in which the two nuclei of the diplokaryon have separated. The sporophorous vesicle is now visible as an envelope surrounding the sporont. Electron-dense material (arrows) at the surface of the sporont appears to be spreading over the plasma membrane. **10.** Sporongy. Three uninucleate and apparently free sporoblasts, together with metabolic products (MP) condensed into one (or a few) discreet clumps. **11.** Uninucleate sporoblasts with a regular oval shape. The metabolic products within the sporophorous vesicle now take the form of tubules (arrow) and pale homogenous spheres (double arrows) as well as the clumps of electron-dense material. Fig. 4–7, Giemsa-stained smears; Fig. 8–11, transmission electron micrographs. Fig. 1–7, bar = 10 μm; Fig. 8–11, bars = 1 μm.

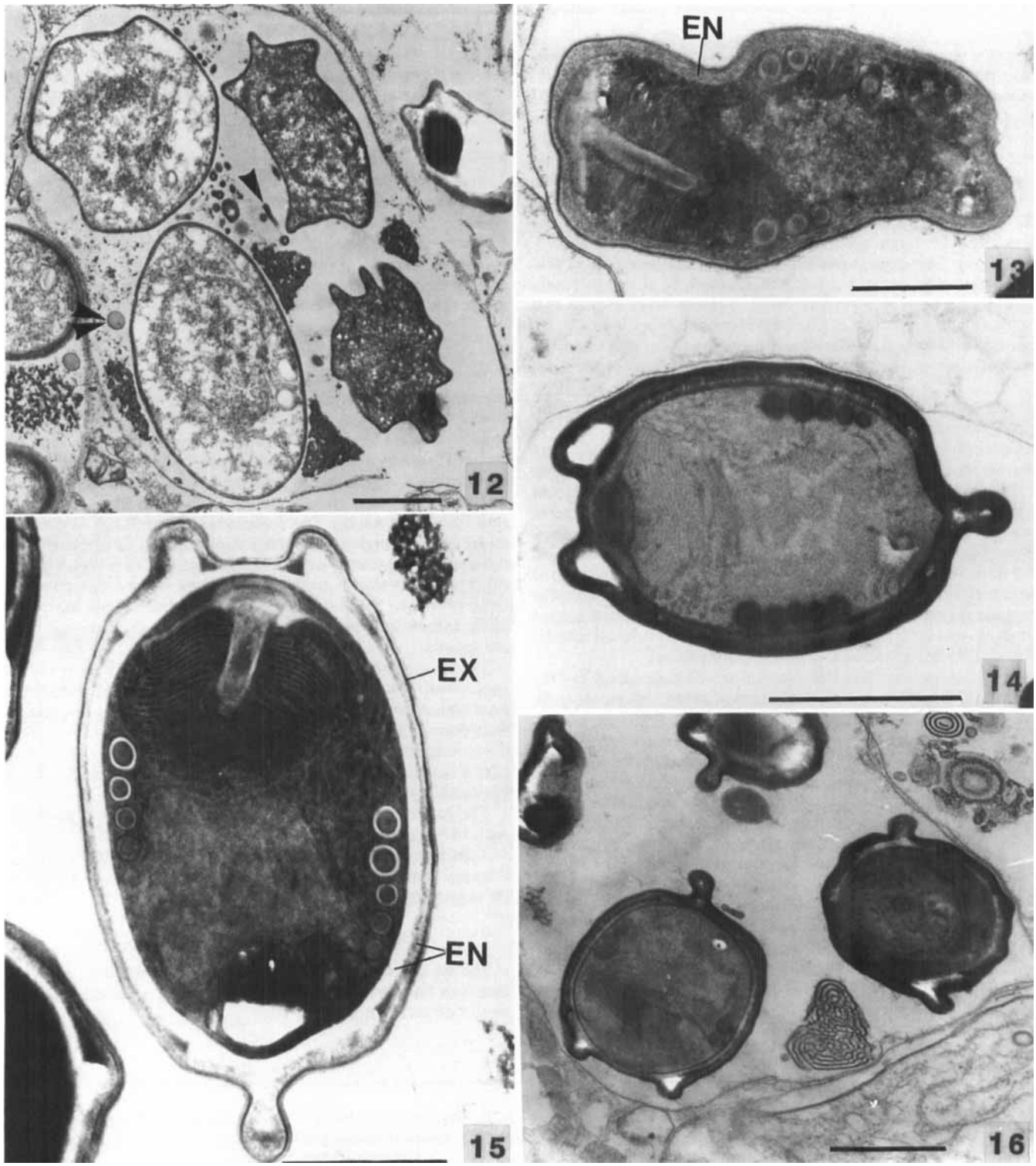

Fig. 12–16. Transmission electron micrographs of *Tricornia muhezae* from *M. africana* larvae. **12.** Ovoid and crenated sporoblasts within a sporophorous vesicle. Tubular metabolic products (arrow) and pale spheres (double arrows) are still present but the large aggregates are less dense. **13.** Maturing sporoblast. Several layers of the future spore wall are visible. EN, endospore. **14.** Immature spore. The shape of the spore is developing and the cytoplasm contains cisternae of endoplasmic reticulum, studded with ribosomes. **15.** Mature spore. The endospore (EN) has resolved into an electron lucent and a moderately electron-dense layer surrounded by the electron-dense exospore (EX). The polar filament is anisofilar, the anterior two coils being thicker than the posterior three. The anterior end is raised into a rim into which all layers of the wall extend. At the posterior end there is a single projection. **16.** Mature spore. The spore on the right is cut in transverse section through the polar filament demonstrating the presence of two lateral projections, suggesting a crest running longitudinally around the spore. Bars = 1 μm.

The genus and species are classified in the family Thelohaniidae Hazard and Oldacre 1975.

Type host. *Mansonia africana.* Infects the cells of the fat body of larvae. Stages were found in adult mosquitoes but could not be linked unequivocally to the larval infection.

Type locality. Muheza and its environs, N.E. Tanzania.

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