

Light and Electron Microscope Studies on Three New Species of Microsporidia from Saltmarsh Mosquitoes in Australia

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SUMMARY

Descriptions are given of three microsporidians found parasitising larvae of culicine mosquitoes in south-east Queensland, Australia. Two, *Duboscqia aediphaga* n.sp. and *Microsporidium lotaensis* n.sp. were recovered from larvae of *Aedes (Ochlerotatus) vigilax* (Skuse), and a third, *Amblyospora pinensis* n.sp., from *Culex sitiens* Wiedemann. The three species were studied by light microscopy and scanning and transmission electron microscopy. We believe this to be the first detailed account of the ultrastructure of a species of *Duboscqia* Perez.

Introduction

During a small-scale search for parasites of mosquito larvae in southeast Queensland, three different microsporidian infections were found in two species of salt-marsh mosquitoes. It was the first record of Microsporidia infecting mosquitoes in Australia and was briefly noted by Kettle and Freebairn [13]. Since then Sweeney et al. [27] have recorded *Amblyospora dyxenoides* infecting *Culex annulirostris* Skuse in Mildura, Victoria, Australia.

The three initial infections comprised *Amblyospora pinensis* n.sp. in larvae of *Culex sitiens* Wiedemann, and *Duboscqia aediphaga* n.sp. and *Microsporidium lotaensis* n.sp. in *Aedes vigilax* (Skuse). Infected larvae of both species of mosquitoes were collected at Dohles Rock on the Pine River, 20 km north of Brisbane and from Lota Creek, 16 km east of Brisbane. *D. aediphaga* and *Amblyospora pinensis* were found at both sites and *M. lotaensis* only at Lota Creek.

Six years later in 1987 one of us (RGP) has found *D. aediphaga* in *Ae vigilax* in 5 coastal localities in tropical North Queensland and *A. pinensis* in *Cx sitiens* in 7, including 4 with *D. aediphaga*.

Material and Methods

Infected larvae were collected in the field, and the parasites studied in fresh preparations, in stained smears, in sections, and by scanning and transmission electron microscopy. The presence of mucous envelopes around spores and sporophorous vesicles

was determined by the Indian ink method of Lom and Vavra [20]. Smears were fixed either in methyl alcohol and stained with Giemsa or in aqueous Bouin's solution and stained with Heidenhain's iron haematoxylin as recommended by Hazard and Oldacre [10]. Fresh spores were examined and photographed using phase contrast.

For transmission electron microscopy small pieces of fat body from infected larvae were fixed at 5 °C, in 5% gluteraldehyde in 0.1 M sodium cacodylate and 0.002 M calcium chloride at pH 7.2. Secondary fixation was in 1% osmium tetroxide in the same buffer solution. Material was then dehydrated by passage through the alcohols, cleared in propylene oxide and embedded in Araldite [21] or Spurr's [26] medium. Ultrathin (gray interference) sections were cut on an LKB ultramicrotome Mark 3, double stained with uranyl acetate and lead citrate and examined under an AEI Corinth electron microscope at an acceleration voltage of 60 kV. For scanning electron microscopy smears were either fixed in the vapour from 2% osmium tetroxide and then air dried before coating or processed as for transmission electron microscopy but after dehydration in alcohol, transferred to amyl acetate and subjected to carbon dioxide critical point drying, the technique recommended by Vavra and Barker [33]. Material was coated with gold and examined under a Cambridge 600 Stereoscan electron microscope at an acceleration voltage of 7.5 kV.

Fresh spores were immobilised in paraffin oil and measured by the photographic technique of Vavra [30]. The terminology of Larsson [16] and Vavra and Sprague [34] will be followed.

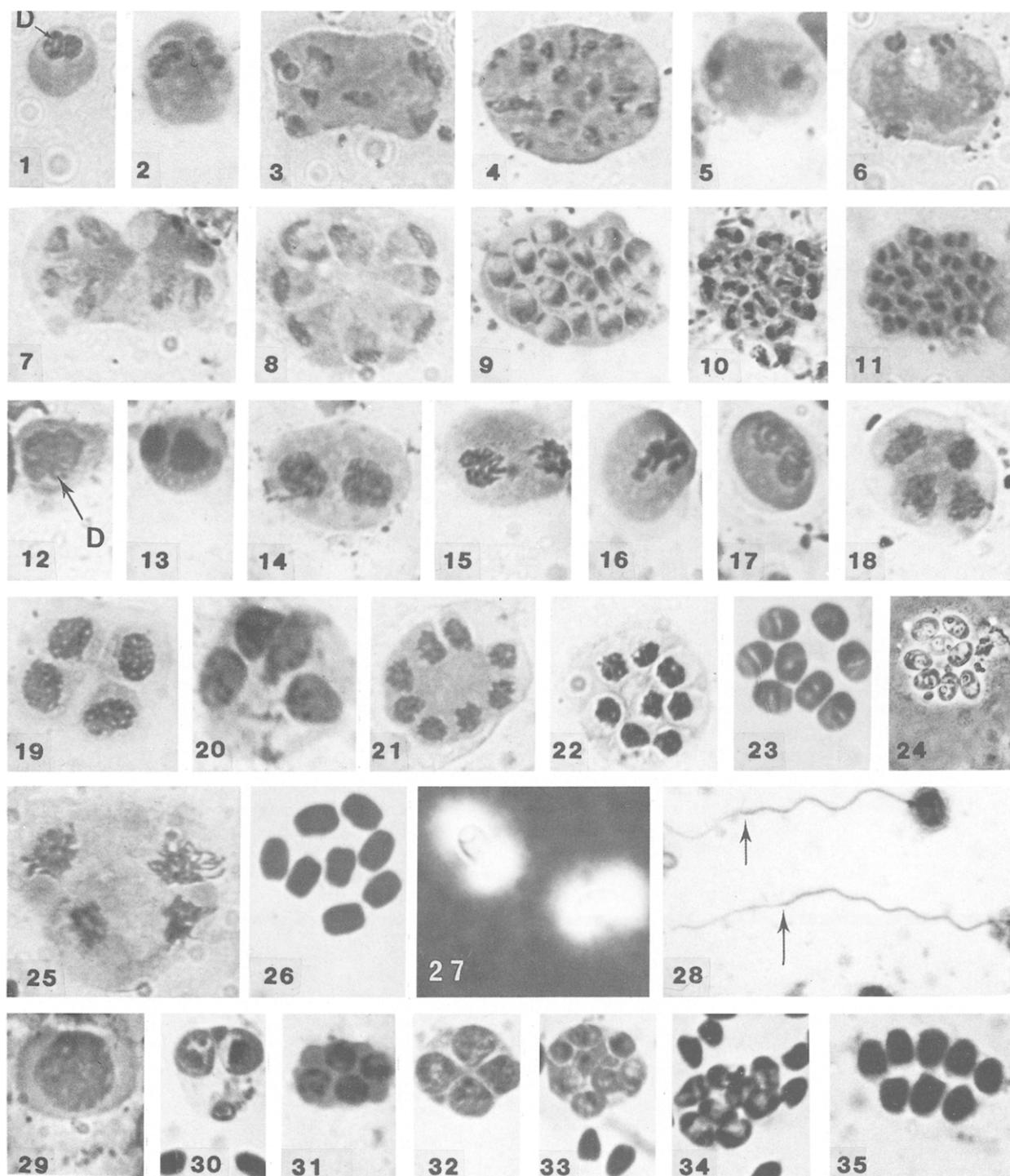
Observations

Heavily infected larvae were readily recognised in the field by their different colouration and behaviour. Larvae

infected with *A. pinensis* and *M. lotaensis* developed opaque white patches in the thorax and abdomen. Larvae infected with *D. aediphaga* developed a reddish coloration in the thorax which, on occasions, was strongly marked, but in N. Queensland some infected larvae have been found with opaque white patches. Infected larvae were more sluggish than healthy ones and spent longer in the larval stage. Larvae with obvious infections either died in

the fourth instar or on pupation. Infected larvae gave rise to abnormal pupae in which the abdomen was greatly swollen and the respiratory horns poorly developed.

Ae vigilax breeds in temporary brackish pools, where it is dependent on high tides flooding the breeding site and stimulating the hatching of larvae from diapausing eggs. The larval stage of infected larvae is protracted and hence the proportion of infected larvae increases as the bulk of



the population completes development and emerges. For that reason it is not possible to give an accurate estimate of the degree of parasitisation but it was very low, not exceeding 1%, comparable with that found by Chapman [7] and Andreadis [1].

Descriptions of new Species

Type Slides: Slides will be deposited with the International Protozoan Type Slide Collection, Smithsonian Institute, Washington, D. C., U.S.A.

Duboscqia aediphaga n.sp.

Light Microscopy

Serial sections of *Ae vigilax* larvae stained with Heidenhain's iron haematoxylin show that *D. aediphaga* develops in the larval fat body. In Giemsa-stained smears sporonts are identifiable by being enclosed in sporophorous vesicles and by their more lightly staining cytoplasm. Meronts develop unenclosed and their cytoplasm stains more deeply.

Merogony. The early meront is bounded by a single unit membrane and has paired nuclei in a diplokaryotic arrangement (Fig. 1). This has been confirmed by transmission electron microscopy (Fig. 37). It gives rise to meronts with two diplokarya (Fig. 2) and then merogonial plasmodia with 8 or 16 diplokarya evenly distributed throughout the cytoplasm (Figs. 3, 4). Presumably this is followed by cytokinesis leading to the release of separate merozoites.

Sporogony. In the binucleate sporont the nuclei are widely separated (Fig. 5). At the 4-nucleate stage the cytoplasm of the sporont becomes constricted in the middle giving rise to two equal portions each containing two widely separated nuclei (Fig. 6). At the 8-nucleate stage the constriction becomes more pronounced and the cytoplasm lobed around the peripheral nuclei (Fig. 7). Sometimes the gap between the two halves is very narrow and the 8 lobes have the appearance of a rosette (Fig. 8). Further nuclear division followed by cytokinesis produces 16 sporoblasts (Fig. 9). At first the developing sporoblasts contain a darkly staining body and cap separated by a very lightly staining "vacuole" (Fig. 9). Later, a linear, reddish

staining structure becomes apparent (Fig. 10). Milner [22] has described a similar structure as the developing polar filament. In other sporoblasts the deeply staining material is U-shaped with the two arms connected by a narrow, less deeply staining strip. They may be more or less equally developed (Fig. 11) or one arm larger than the other. More mature sporoblasts stain deeply with Heidenhain's haematoxylin and show a narrow clear zone between the main body and the crescent-shaped cap, similar in appearance to fresh spores under phase contrast.

Spore. Fresh spores are ovoid to ellipsoid in shape, have no mucous envelope, and measure $5.6 \times 2.6 \mu\text{m}$ (Table 1). Fixed, stained spores are slightly shorter, measuring $4.9 \times 2.6 \mu\text{m}$ (Table 1).

Sporophorous vesicles. In fresh preparations sporophorous vesicles are ovoid, measuring $17.0 \times 11.5 \mu\text{m}$ (Table 1). In fixed preparations they appear larger, having all the spores in the one plane, and measure $20.3 \times 13.8 \mu\text{m}$. They lack the delicate needle-like spines characteristic of *Trichoduboscqia* Léger [18]. Sixty sporophorous vesicles have been examined and all contained 16 spores but vesicles with 8 spores have been seen rarely.

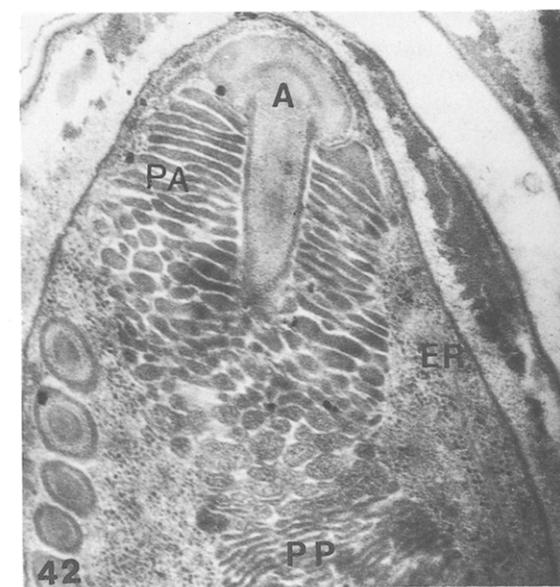
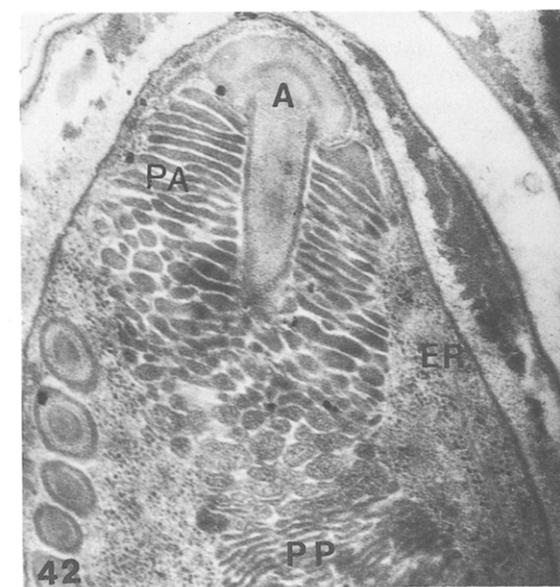
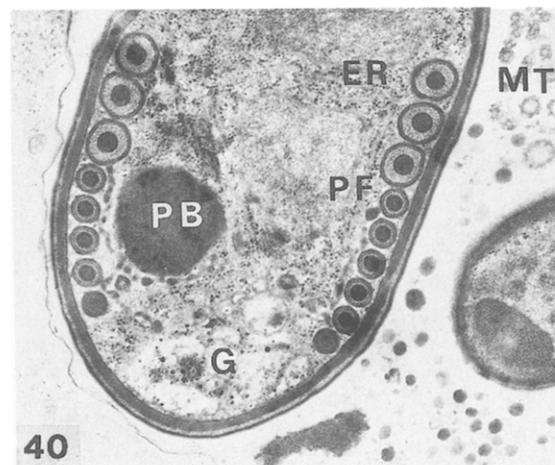
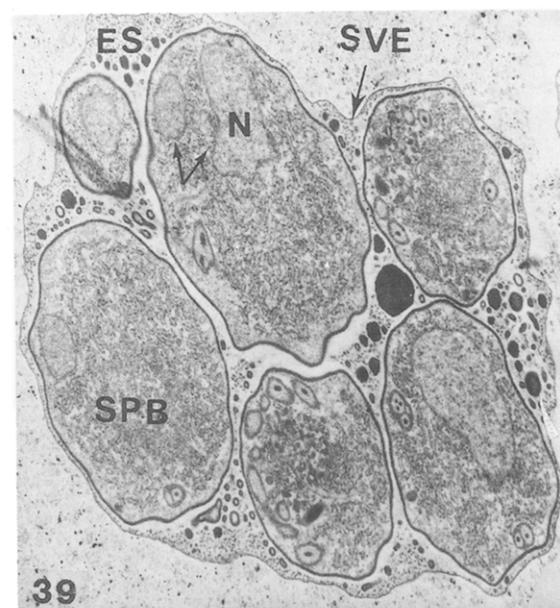
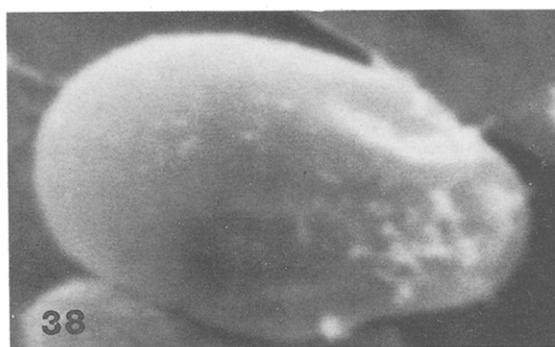
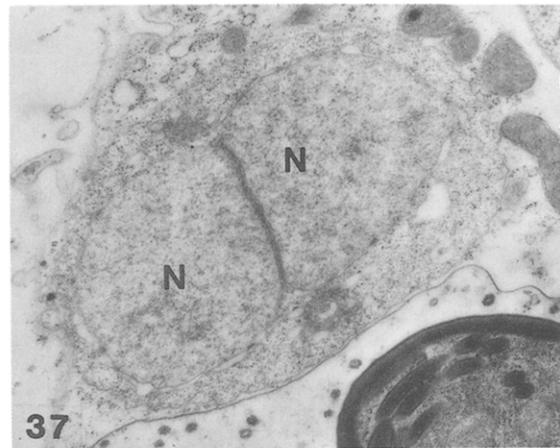
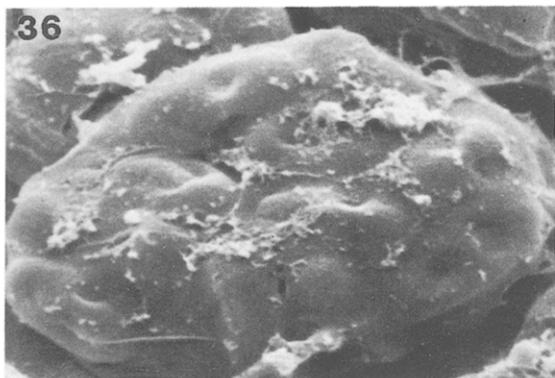
Electron Microscopy

Spore ultrastructure. Spores fixed in osmium vapour show a smooth anterior cap, which is presumably the polar cap seen in stained spores. When spores are prepared by critical point drying the anterior cap is less obvious and the spore surface is either finely corrugated or apparently smooth (Fig. 38).

The spore wall consists of a relatively thick, electron lucent endospore and a thin multilayered exospore (Fig. 41). The latter is wrinkled or finely corrugated and composed of five layers, of which the outer three are thin and the inner two are thicker. The innermost, electron dense layer is incomplete. The spore contents are surrounded by a thin unit plasma membrane.

The detailed ultrastructure of the spore is similar to that of other Microsporidia. The anchoring apparatus is located anteriorly and extends posteriorly through the polaroplast as the manubroid part of the polar filament (Fig. 42). In a well-developed spore the polaroplast is composed of broad lamellae anteriorly and narrow, closely packed lamellae posteriorly, separated by an intervening

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- ◀ Figs. 1–11. *D. aediphaga*. – Fig. 1. Meront with diplokaryon (D). – Fig. 2. – Meront with two diplokarya. – Figs. 3–4. Merogonial plasmodia with 8- and 16-diplokarya. – Fig. 5. Binucleate sporont. – Fig. 6. 4-nucleate sporont. – Figs. 7–8. 8-nucleate sporogonial plasmodia. – Figs. 9–11. 16 developing sporoblasts in sporophorous vesicles.
 Figs. 12–27. *A. pinensis*. – Fig. 12. Meront with diplokaryon (D). – Figs. 13–14. Meronts with 2 nuclei. – Fig. 15. Meront with 2 nuclei undergoing karyokinesis. – Fig. 16. Uninucleate sporont in sporophorous vesicle. – Fig. 17. Sporont with 2 nuclei. – Fig. 18. 4-nucleate sporogonial plasmodium. – Fig. 19. 4-nucleate sporont with cytokinesis almost complete. – Fig. 20. 4-nucleate with cytokinesis completed. – Fig. 21. 8-nucleate sporogonial plasmodium. – Fig. 22. Developing sporoblasts. – Fig. 23. Nearly mature spores. – Fig. 24. Sporophorous vesicle with 8 spores under phase contrast. – Fig. 25. 4-nucleate merogonial plasmodium. – Fig. 26. Mature spores. – Fig. 27. Mature spores with surrounding mucous envelopes against a background of Indian ink.
 Figs. 28–35. *M. lotaensis*. – Fig. 28. Ejected polar filaments displaying anisofilar structure. Change in diameter arrowed. – Fig. 29. Early sporont in sporophorous vesicle. – Fig. 30. Binucleate sporonts. – Fig. 31. 4-nucleate sporont with cytokinesis complete. – Fig. 32. 4-nucleate 'rosette' form of sporont. – Fig. 33. 8 sporoblasts in sporophorous vesicle. – Fig. 34. Young spores. – Fig. 35. Mature spores. All Figs. $\times 1500$. – Figs. 1–22, 25, 28, 29 and 31 from preparations stained with Giemsa. – Figs. 23, 26, 30, 32–35 stained with Heidenhain's haematoxylin.



vesicular zone (Fig. 42). This is the reverse of the usual arrangement in which the anterior lamellae are more closely packed. The anisofilar polar filament is coiled posterolaterally and abruptly constricted in the first half of its length (Fig. 40). The number of wide coils ranges from 2.5 (i.e. 2 on one side and 3 on the other) to 4, and narrow coils from 4 to 5.5 with a total of 7.5 to 8.5 ($n = 9$). The most mature spore in which the details of the polar filament could be observed had 3.5 wide coils and 6.5 narrow coils totalling 10. The angle of tilt [6] was low, about 80°.

The electron dense posterior body is probably spherical, being circular in outline in section (Fig. 40). Sometimes there are two posterior bodies. The Golgi apparatus is situated posterior to the posterior body. Rough endoplasmic reticulum is prominent within the cytoplasm of the spore. The posterior vacuole is not well developed. The nucleus is bounded by a double membrane, which is granular on its cytoplasmic surface.

Sporophorous vesicle envelope and episporontal space. Under the scanning electron microscope sporophorous vesicles appear as flattened ovoid or thickened disc-shaped bodies (Fig. 36). The envelope of the sporophorous vesicle is of unit type with granules attached to its inner surface. It loosely surrounds the spores (Fig. 39). The corresponding sporoblast membranes have granules attached to their outer surfaces, adjoining the episporontal space. The contents of the latter are heterogeneous including dense granules, electron-dense microtubules, much larger tubules or vesicles (Fig. 40) and obvious electron-dense bodies (Fig. 39), which vary in size and are probably ovoid in shape. In section they have evenly curved outlines.

Diagnosis

Duboscqia aediphaga n.sp.

Host: *Aedes (Ochlerotatus) vigilax* (Skuse), the salt-marsh mosquito.

Type locality: Dohles Rock, Pine River, south-east Queensland, Australia.

Site of infection: Fat body of larva, established by sectioning infected larvae.

Vegetative stages: Diplokaryotic meronts give rise to plasmodia which form sporonts by budding.

Sporulation stages: Sporonts in larvae contain 2, 4, 8 or 16 nuclei. Each sporont gives rise to 16 sporoblasts within a sporophorous vesicle. Rarely only 8 spores are formed.

Spores: Within the larva, ovoid to ellipsoid uninucleate spores are formed measuring $5.6 \pm 0.3 \times 2.6 \pm 0.1 \mu\text{m}$ ($n = 25$) in fresh preparations and $4.9 \pm 0.4 \times 2.6 \pm 0.2$

μm ($n = 25$) in fixed material. They have a thick endospore and thin exospore. The anisofilar polar filament has 7.5 to 8.5 coils, of which 2.5 to 4 are wide and 4 to 5.5 narrow, with an angle of tilt of about 80°.

In Larsson's [17] key to the genera of Microsporidia this species keys out to *Duboscqia* by its possession of 16 oval spores in a sporophorous vesicle without appendages. According to Sprague [24] 11 species have been referred to *Duboscqia* Perez but he rejects 6 of these as not being Microsporidia. The remaining 5 include two parasites of termites, two of Crustacea and one of a dipteran, *Chironomus plumosus* L. *D. aediphaga* is the first member of the genus to be described from the Culicidae.

Amblyospora pinensis n.sp.

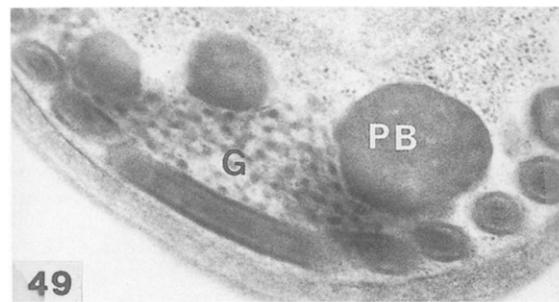
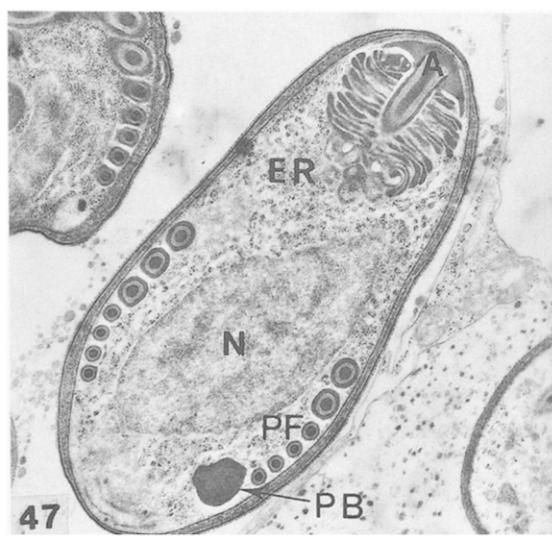
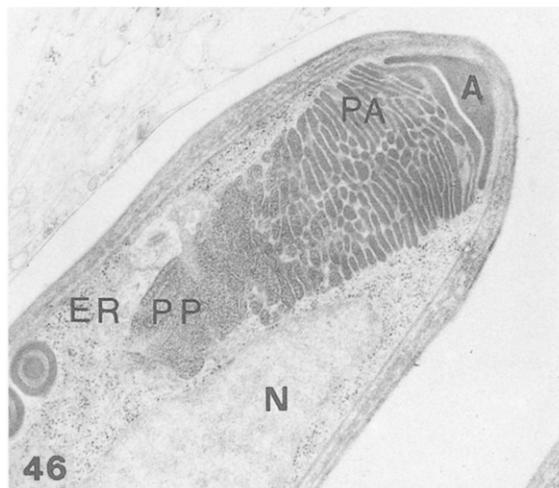
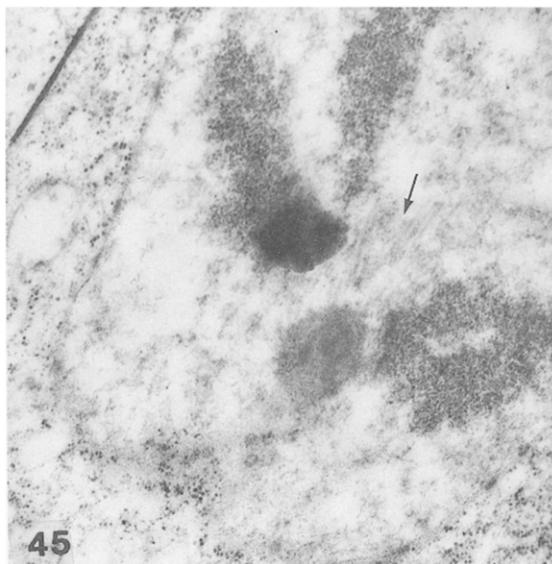
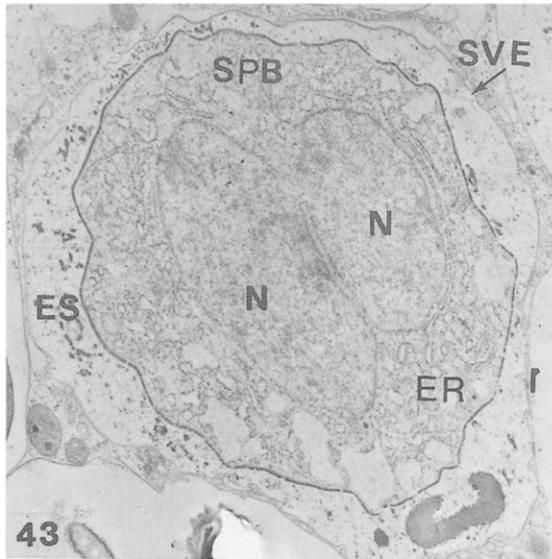
Light Microscopy

Merogony. In Giemsa stained preparations paired nuclei in diplokaryotic arrangement occupy the greater part of the meront (Fig. 12). They separate (Figs. 13, 14) and at karyokinesis chromosomal strands become prominent (Figs. 15, 25). Later they lose their identity when the boundaries of the nuclei become more distinct. Four and presumably 8-nucleate merogonial plasmodia are formed (Fig. 25).

Sporogony. In Giemsa stained preparations only a single nucleus is recognisable in the early sporont (Fig. 16). At karyokinesis strands of deeply staining chromosomal material are observable. The nucleus divides to form two and 4-nucleate sporonts (Figs. 17, 18). At this stage the cytoplasm usually becomes deeply lobed with very narrow connections, the 4 lobes being typically arranged in a cruciform shape (Fig. 19). Sometimes cytokinesis may proceed to completion and separate uninucleate bodies appear in the sporophorous vesicle at the 4-nucleate stage (Fig. 20). Although separated, these uninucleate bodies probably divide to produce two sporoblasts. Sometimes cytokinesis may be delayed and 4- and 8-nucleate sporogonial plasmodia are formed with only modest lobing (Fig. 21). In all cases at the 8-nucleate stage 8 sporoblasts are formed in which initially the only differentiation is the reddish staining nucleus (Fig. 22). Later a concentrated, deeply staining dark blue body is prominent in each sporoblast. Mature, or nearly mature, spores stained with Heidenhain's haematoxylin are either uniformly dark (Fig. 26) or stain deeply at one end with the remaining two-thirds being paler except for a narrow transverse band about midway (Fig. 23).

Sporophorous vesicle. Numerous sporophorous vesicles have been examined and all contained eight spores. They

◀ Figs. 36–42. Electron micrographs of *D. aediphaga*. — Fig. 36. Sporophorous vesicle $\times 5000$. — Fig. 37. Meront with diplokaryon $\times 15500$. — Fig. 38. Critical point dried spore $\times 16000$. — Fig. 39. Early sporoblasts in sporophorous vesicle with solid metabolic bodies in the episporontal space and arched nucleus cut twice (arrowed) in one sporoblast $\times 9000$. — Fig. 40. Young spores with anisofilar polar filament and posterior body $\times 23000$. — Fig. 41. Mature spores with thick endospore and thin exospore $\times 8000$. — Fig. 42. Young spore showing anchoring apparatus and polaroplast $\times 38000$. A = anchoring apparatus; EN = endospore; ER = endoplasmic reticulum; ES = episporontal space; EX = exospore; G = Golgi apparatus; MT = microtubules; N = nucleus; PA = anterior polaroplast; PB = posterior body; PF = polar filament; PP = posterior polaroplast; SPB = sporoblast; SVE = envelope of sporophorous vesicle.



are ovoid bodies (Fig. 24) which measure 14.9 by 12.4 µm in the fresh state and slightly less (14.4 by 11.1 µm, Table 1) in fixed preparations.

Spores. Spores are barrel-shaped with convex sides and truncated ends (Fig. 26). Fresh spores measure 5.9 by 3.8 µm (Table 1), and fixed, stained spores 4.5 by 2.9 µm. The prominent mucous envelope, which surrounds fresh spores, is weakly developed in a narrow band about mid-length (Fig. 27). In preparations stained with Heidenhain's haematoxylin a polar cap cannot be distinguished (Fig. 26).

Electron Microscopy

Merogony. The early meront has a thinner plasmalemma than the early sporont and is not surrounded by a sporophorous vesicle. A typical diplokaryon is present in which aggregations of electron dense material are evenly distributed throughout the nuclei giving a dappled appearance (Fig. 44). In the sporont the few large aggregations are unevenly distributed throughout the nucleus (Fig. 43).

Sporogony. The early sporont is surrounded by the envelope of the sporophorous vesicle. Its cell membrane is thicker and electron denser than the envelope. The nucleus is very large and appears to be in two parts narrowly separated for much of their adjacent surfaces and united over a short length (Fig. 43). The nucleus is heterogeneous with amorphous dark areas within. The cytoplasm is vacuolated and penetrated by tubules. Loosely fitting continuous membranes occur in the vacuoles and they together with the tubules probably represent the developing endoplasmic reticulum and in other areas rough endoplasmic reticulum is distinguishable.

Spore. Air dried spores show strong longitudinal ribs or abundant finer transverse corrugations, but when prepared by critical point drying the exospore appears quite smooth.

Spore ultrastructure. In the mature spore the wall is finely corrugated and composed of a broad electron lucent endospore and a thinner exospore. In maturing spores, before the endospore has been differentiated, the spore wall is composed of seven thin, alternately light and dark granular layers external to the cytoplasmic membrane surrounding the spore contents (Fig. 46). Even when the endospore is well differentiated several thin layers can be made out in the exospore including an outer granular layer, a prominent electron dense layer, an indistinct lucent layer and a thick inner amorphous layer. The endospore is thinner anteriorly adjacent to the anchoring apparatus.

The manubroid part of the polar filament extends posteriorly from the convex anchoring disc through the hinge.

In the anterior half of the spore the polar filament is surrounded by the polaroplast, which is lamellate anteriorly and vesicular posteriorly (Fig. 46). In the mature spore the lamellae of the polaroplast are separated by a narrow pale band, bisected by a very thin electron dense layer. The anisofilar polar filament is coiled posterolaterally (Fig. 47) and averages seven coils of which 2.7 are wide and 4.3 narrower ($n = 15$). The ranges for wide and narrow coils are 2 to 3.5 and 3.5 to 5 respectively.

A single, large, prominent nucleus fills much of the developing spore (Fig. 46) but with the development of cytoplasmic organelles it becomes compressed. At all stages the nucleus is heterogeneous with conspicuous, amorphous electron dense areas within it. In the cytoplasm there is a well developed rough endoplasmic reticulum.

The Golgi apparatus can sometimes be distinguished at the posterior end of the spore. It may take the form of concentric oval electron dense layers (Fig. 48) becoming vesicular with darker granules posteriorly or an anastomosing meshwork of vesicles with scattered electron dense granules (Fig. 49). There are other bodies in the posterior half of the spore which are probably secreted by the Golgi apparatus and for which the name posterior body appears inappropriate. One, two or three rounded, probably spherical, electron dense bodies can be found posteriorly (Figs. 47, 49). In addition, there may be a number of other angular, electron dense blocks occupying a substantial part of the spore posteriorly. Typically these blocks appear as discrete entities separated by an antero-posterior fissure and further separated by transverse fissures. It is as if a single block has been fractured both longitudinally and transversely (Fig. 44). The posterior vacuole appears very late in development being recognisable at first as a paler area with scattered electron dense granules. Later in the mature spore the posterior vacuole is lucent.

Episporontal space. The episporontal space contains large vacuolated electron dense bodies (Fig. 43) and small particles which are not obviously tubular.

Diagnosis

Amblyospora pinensis n.sp.

Host: *Culex sitiens* Wiedemann, the saltmarsh culex.

Type locality: Dohles Rock, Pine River, south-east Queensland, Australia.

Site of infection: Fat body of larva, established by sectioning infected larvae.

Vegetative stages: Diplokaryotic meronts give rise to plasmodia which form other meronts and presumably sporonts by budding.

◀ Figs. 43–49. Electron micrographs of *A. pinensis*. – Fig. 43. Early sporont $\times 9000$. – Fig. 44. Diplokaryon in meront, and dense bodies in maturing spore (arrowed) $\times 9000$. – Fig. 45. Nucleus of developing sporont showing synaptonemal complexes (arrowed) $\times 30000$. – Fig. 46. Maturing spore showing polaroplast and anchoring apparatus $\times 22300$. – Fig. 47. Maturing spore $\times 14500$. – Figs. 48–49. Golgi apparatus in maturing spores $\times 38000$. A = anchoring apparatus; ER = endoplasmic reticulum; ES = episporontal space; G = Golgi apparatus; N = nucleus; PA = anterior polaroplast; PB = posterior body; PF = polar filament; PP = posterior polaroplast; SPB = sporoblast; SVE = envelope of sporophorous vesicle.

Sporulation stages: Sporonts in larvae contain 2, 4 or 8 nuclei. Each sporont gives rise to 8 sporoblasts within a sporophorous vesicle.

Spores: within the larva, barrel-shaped uninucleate spores with convex sides and truncated ends. Fresh spores measure $5.9 \pm 0.3 \times 3.8 \pm 0.2 \mu\text{m}$ ($n = 25$), and fixed spores $4.5 \pm 0.3 \times 2.9 \pm 0.3 \mu\text{m}$ ($n = 25$, Table 1). They possess a well developed endospore (125–250 nm) and a thinner, but still substantial exospore (60–160 nm). The anisofilar polar filament is in one layer with 7 coils of which 2 to 3.5 are wide and 3.5 to 5 are narrow, with an angle of tilt of about 70°.

Microsporidium lotaensis n.sp.

Light Microscopy

Merogony. No clearly identifiable stages in merogony were recognisable in stained preparations. This is compatible with the advanced stage of infection in the material examined. Diplokaryotic meronts were observed in transmission electron microscopy (Fig. 52).

Sporogony. The infected *Ae vigilax* larvae available for study yielded many spores but few developing stages. The sporont develops in a sporophorous vesicle, within which deeply staining metabolic bodies can be seen. They disappear later during spore formation. In the early sporont the nuclei are associated in a diplokaryon (Fig. 54) and at all stages they are inconspicuous showing only moderate contrast with the cytoplasm (Fig. 29). At the binucleate stage the sporont becomes deeply divided into two equal lobes (Fig. 30). After the next nuclear division 4 lobes are formed and often appear in the familiar cruciform shape with the lobes being united by narrow connections (Fig. 32). Sometimes the four subdivisions of the sporont are completely separated (Fig. 31). It is not known whether these prosporoblasts proceed directly to the sporoblast stage or whether they divide again and form two sporoblasts. The presence of macrospores (Fig. 50) suggests that some separated bodies become sporoblasts. Sometimes the 4 prosporoblasts are of similar size to spores and it is considered that they become sporoblasts without further division. This possibility cannot be confirmed because the sporophorous vesicle does not persist until the spores are mature. Sporophorous vesicles have been found with 8 developing sporoblasts (Fig. 33) but never with fully mature spores (Figs. 28, 35). Melanised spores have been observed, indicating some host response to infection.

Sporophorous vesicle. The sporophorous vesicle is short-lived and was not observed in fresh preparations or under the scanning electron microscope. In stained, fixed

Table 1. Measurements (mean \pm standard deviation) of sporophorous vesicles and spores of *D. aediphaga*, *A. pinensis* and *M. lotaensis* using Vavra's [30] photographic technique

	n	Length μm	Breadth μm
(a) <i>Duboscqia aediphaga</i>			
Sporophorous vesicle fresh	5	17.0 ± 1.2	11.5 ± 0.6
Sporophorous vesicle fixed	30	20.3 ± 1.1	13.8 ± 1.0
Spore fresh	25	5.6 ± 0.3	2.6 ± 0.1
Spore fixed	25	4.9 ± 0.4	2.6 ± 0.2
(b) <i>Amblyospora pinensis</i>			
Sporophorous vesicle fresh	4	14.9 ± 0.5	12.4 ± 0.6
Sporophorous vesicle fixed	25	14.4 ± 1.4	11.1 ± 1.0
Spore fresh	25	5.9 ± 0.3	3.8 ± 0.2
Spore fixed	25	4.5 ± 0.3	2.9 ± 0.3
(c) <i>Microsporidium lotaensis</i>			
Sporophorous vesicle fixed	25	13.7 ± 1.5	11.4 ± 1.4
Spore fresh	25	6.1 ± 0.2	3.7 ± 0.2
Spore fixed	25	4.5 ± 0.3	3.1 ± 0.2

preparations sporophorous vesicles measured 13.7 by 11.4 μm (Table 1). They were not common but all those examined contained 8 spores.

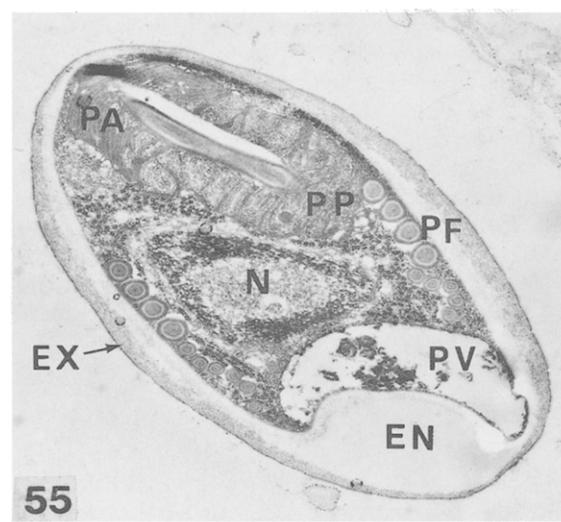
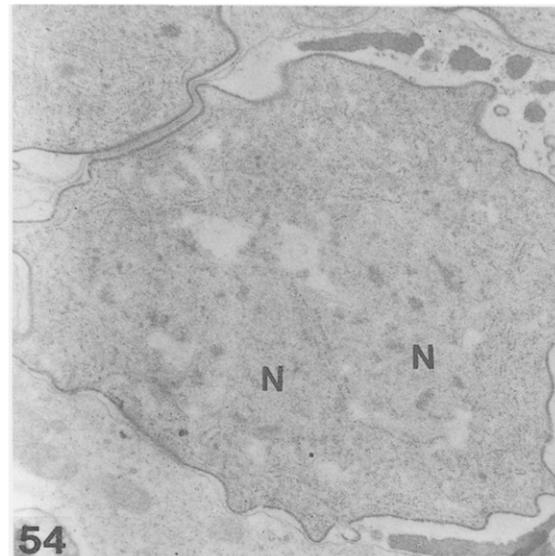
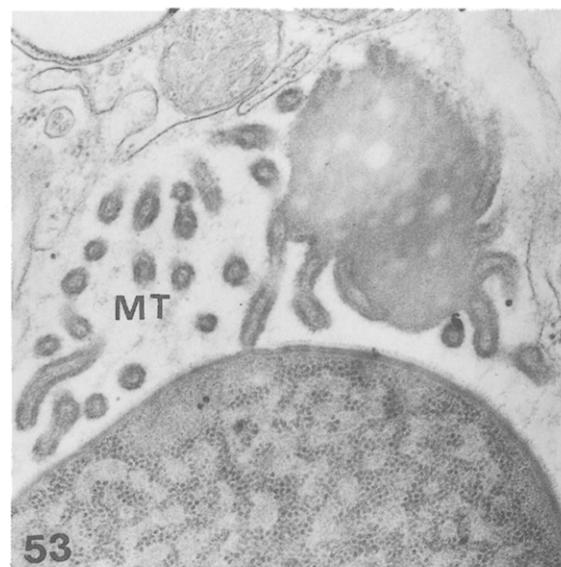
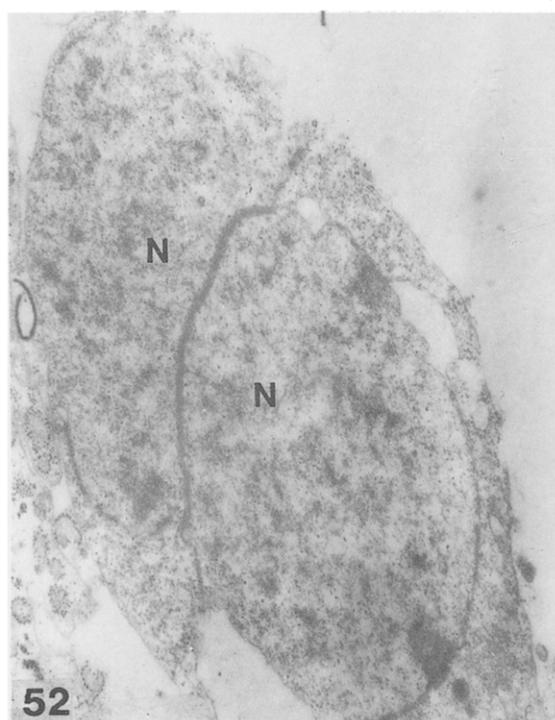
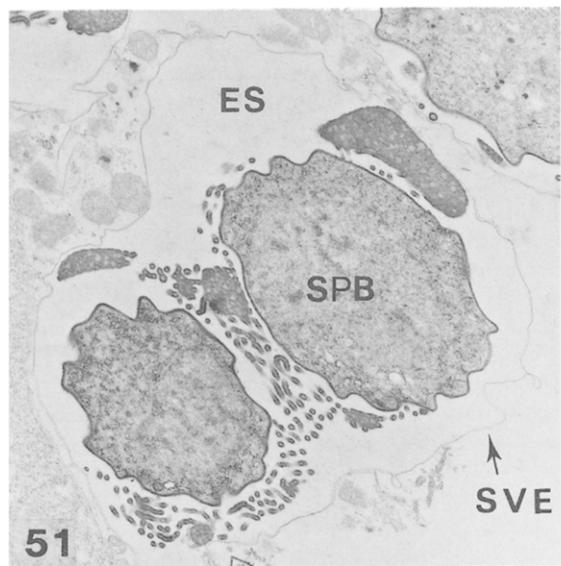
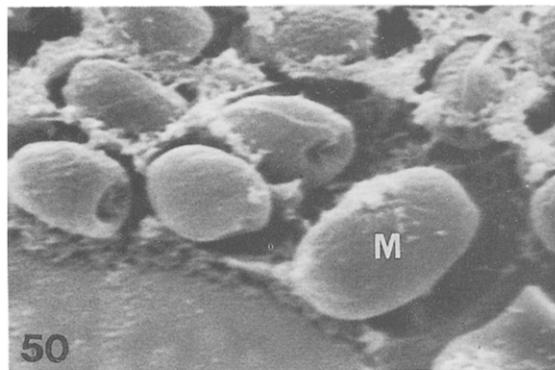
Spore. Spores are truncate at one end and bluntly rounded at the other end (Fig. 35). Fresh spores measure 6.1 by 3.7 μm and fixed, stained spores 4.5 by 3.1 μm (Table 1). There is no mucous envelope surrounding the fresh spore. In stained preparations a polar cap was not seen.

Electron Microscopy

Spore. When spores are prepared by critical point drying the exospore appears smooth with fine corrugations (Fig. 50). There is a pronounced invagination at one end which corresponds with the truncated end of the spore in stained preparations, where there is an indication of a concealed depression (Fig. 35). A small number of larger spores (macrospores) are present (Fig. 50).

Spore ultrastructure. The maturing spore is characterised by early development of the posterior vacuole and the apparent hypertrophy of the adjacent endospore (Fig. 55). The posterior vacuole arises as an irregularly shaped membrane-bound space almost devoid of electron dense particles. Later, electron dense particles appear in it and form distinct clumps. Adjacent to the posterior vacuole there develops a broad electron lucent zone. Its development is accompanied by the posterior vacuole becoming concave. In the mature spore the spore wall is invaginated into the

Figs. 50–55. Electron Micrographs of *M. lotaensis*. — Fig. 50. Spores prepared by critical point drying $\times 4600$. — Fig. 51. Early sporonts showing large metabolic bodies and tubules in the episporontal space $\times 9000$. — Fig. 52. Meront with diplokaryon $\times 20000$. — Fig. 53. Association of metabolic bodies and tubules $\times 38500$. — Fig. 54. Paired nuclei in early sporont $\times 13000$. — Fig. 55. Nearly mature spore $\times 17500$. EN = endospore; ES = episporontal space; EX = exospore; M = macrospore; MT = microtubules; N = nucleus; PA = anterior polaroplast; PF = polar filament; PP = posterior polaroplast; PV = posterior vacuole; SPB = sporoblast; SVE = envelope of sporophorous vesicle.



concavity of the posterior vacuole and the broad electron lucent zone disappears. This zone could arise from hypertrophy of the endospore which, being composed of chitin, is electron lucent and cannot be distinguished in transmission electron microscopy from empty space. If the zone is indeed endospore then during the invagination of the spore wall the greater part of it would need to be absorbed.

The polar filament is attached to the anchoring apparatus from which it is continued posteriorly as the manubroid part surrounded by a lamellate polaroplast anteriorly and a vesicular polaroplast posteriorly. The polar filament is anisofilar (Fig. 55) with 8.5 to 9.5 coils of which the first 4 to 5 are wide and the distal 4 to 5.5 are narrow. In some fixed and stained preparations, spores have ejected their polar filaments and the anisofilar structure of the polar filament is clearly visible with the basal portion being much broader than the distal part (Fig. 28).

The nucleus is heterogenous with amorphous aggregations of electron dense material within it. In both transmission electronmicrographs and stained preparations there is a lack of contrast between nucleus and cytoplasm. Rough endoplasmic reticulum is well developed.

Sporophorous vesicle and episporontal space. At the early sporont stage the episporontal space contains abundant metabolic bodies in the form of tubules and more solidly compact bodies (Fig. 51). The latter may be electron dense to varying degrees (Fig. 51) or vacuolated with electron lucent areas. The tubules, which are closely associated with the compact bodies (Fig. 53), may have uniform contents or be broader and have tubular elements within.

Diagnosis

Microsporidium lotaensis n.sp.

Host: *Aedes vigilax*, the saltmarsh mosquito.

Type locality: Lota Creek in the Brisbane metropolitan area, south-east Queensland.

Site of infection: Fat body of larva by observation but not confirmed by sectioning due to lack of material.

Vegetative stages: Diplokaryotic meronts are present.

Sporulation stages: Each sporont gives rise to 4 to 8 sporoblasts within a sporophorous vesicle.

Spores: Within the larva, broadly conical uninucleate spores with bluntly rounded anterior ends and truncate posterior ends, measuring $6.1 \pm 0.2 \times 3.7 \pm 0.2 \mu\text{m}$ ($n = 25$) in fresh preparations and $4.5 \pm 0.3 \times 3.1 \pm 0.2 \mu\text{m}$ ($n = 25$) in fixed material. They possess a well developed endospore and a thinner exospore. The anisofilar polar filament has 8.5 to 9.5 coils, of which 4 to 5 are wide and 4 to 5.5 are narrow, with an angle of tilt of 60° in the only suitable preparation.

Discussion

Many species of Microsporidia have been recorded from mosquitoes. Sprague [24] listed 101 of which 46 are

named and 55 are identified only to genus. The greatest number (75, 28 named) are in the Amblyosporidae, as recognised by Weiser [36], and small numbers (named species) in other families: Pleistophoridae 9(4); Thelohaniidae 6(4); Nosematidae 4(4); and 7(6) are unclassified and put in the genus *Microsporidium* Balbiani.

Little work has been done on Microsporidia of Australian mosquitoes either in Australia or elsewhere in their geographic range. In south-east Australia Sweeney et al. [27] followed the development of *Amblyospora dyxenoides*, a parasite of *Culex annulirostris* Skuse in its copepod intermediate host. In the New Hebrides Laird [15] attributed an infection in the same species to *Thelohania opacita* Kudo, an identification which is almost certainly incorrect because the parasite is described as having only four spores in the sporophorous vesicle, which would remove it from *Thelohania* Henneguy and place it nearer to *Gurleya* Doflein. Vavra et al. [32] described *Amblyospora indicola* Vavra, Bai and Panicker from *Cx sitiens* in Pondicherry, S. E. India.

Duboscqia aediphaga

Sprague [25] includes 10 families in the suborder Pan-sporoblastina, one of which is the Duboscqiidae containing the genera *Duboscqia* and *Trichoduboscqia* associated by their producing 16 spores within a sporophorous vesicle. Weiser [37] recognises only 4 families in his equivalent category, Pleistophoridae, and includes these two genera with 6 others in the Thelohaniidae, characterised by having uninucleate, thin walled spores with direct peroral infectivity, and an isofilar polar filament [36, 37]. The spores of *D. aediphaga* are uninucleate, but differ in being thick walled and having an anisofilar polar filament.

The only other work known to us on the ultrastructure of *Duboscqia* is an electron-micrograph in a general account of the ultrastructure of microsporidian spores by Vavra [31]. It depicts a longitudinal section through the posterior portion of a young spore of a *Duboscqia* sp. from *Diacyclops* sp. showing the usual structures together with an anisofilar polar filament of 2 wide and 8 narrow coils similar to that described here for *D. aediphaga*. The polar filament of *Trichoduboscqia epeori*, Leger, the only species in the genus, is isofilar with 7 coils [5]. This difference supports Batson's [5] suggestion that these two genera may not be closely related. They have been associated because they produce 16 spores in a sporophorous vesicle but this is variable. *T. epeori* often produces 32 spores [5] and *D. aediphaga* rarely produces 8 spores. In addition, the sporophorous vesicle of *T. epeori* bears needle-like appendages; a fibrillar network occupies the episporontal space; and the spores are pyriform [5]. In *D. aediphaga* there are no appendages; the episporontal space contains microtubules and crystalline bodies; and the spores are oval.

Larsson [16] classified Microsporidia on their ultrastructure and included *Trichoduboscqia* in the *Culicosporella* line of group 4 (p. 376) but did not consider the position of *Duboscqia* because its cytology was unknown. Some of the information required is now available for

D. aediphaga: exospore – type IID with a thickness of 85 nm; polaroplast – type IV; anisofilar polar filament in a single layer with wide coils (250 nm) and narrow coils (160 nm), showing in cross section layers IA, IB, IC, ID, II, III and IV; angle of tilt 80°; sporophorous vesicle – type V with inclusions type D (tubules) and E (crystalline), and envelope thickness (< 10 nm). These characters place *D. aediphaga* in group 4 close to the *Parathelohania* line from which it differs mainly by its type IV polaroplast, compared to type I. When the characteristics of *D. aediphaga* are compared with those of other microsporidian genera in Larsson's [17] table the affinities of *D. aediphaga* are closest to *Parathelohania* Codreanu and the octosporous phase of *Vairimorpha* Pilley. He lists *Duboscqia* as having a single nucleus in the meront, sporont and spore. *D. legeri* Perez has a uninucleate meront and *D. chironomi* Voronin a diplokaryon in the sporont [24]. *D. aediphaga* has a diplokaryotic meront (Figs. 1, 37). The appropriate position for *D. aediphaga* will not be resolved until its full developmental cycle is known.

The spores of *D. chironomi* have a posterior vacuole at the broad end [35], and those of *D. coptotermi* Kalavati and Narasimhamurti a posterior vacuole and an anterior polar cap [12]. In *D. aediphaga* the apparent vacuole is placed immediately posterior to the polar cap at the anterior end of the spore. Lom and Vavra [20] describe the spore of *Duboscqia* sp. as having a wide mucous coat, which is absent in *D. aediphaga*. Kudo [14] states that *D. legeri* has no discernible polar cap, which is present in *D. aediphaga*. He refers to the polar filament as being identifiable in Giemsa stained preparations as a longitudinal linear structure in the middle region of the spore. A similar structure is recognisable in *D. aediphaga*. Kudo [14] also states that the meronts are enclosed in a delicate membrane. No such membrane was detected in *D. aediphaga*. The mature spores of *D. aediphaga* are of similar size to those of other species of *Duboscqia* except those of *D. sidae* Jirovec which are considerably smaller measuring only 3 × 1.2 to 1.6 µm [11].

Amblyospora pinensis

Amblyospora spp. parasitic in mosquitoes are dimorphic in sporulation producing thick-walled monokaryotic octospores in sporophorous vesicles, mostly in male larvae, and thin-walled diplokaryotic free spores in adult females [10, 16]. The latter pass by transovarian transmission to the next generation but this is insufficient to maintain *Amblyospora* spp. in *Culex salinarius* Coquillett [3, 4] or in *Aedes cantator* (Coquillett) [1]. Although Andreadis [2] obtained some sporadic horizontal transmission of *Amblyospora* sp. in *Aedes stimulans* (Walker) octospores are not generally infective to mosquitoes, and an alternative method of parasite survival is required. Such a route has been found for *Amblyospora dyxenoides* infecting *Cx annulirostris* in which octospores infect *Mesocyclops albicans*, a copepod, and produce spores infective to *Cx annulirostris* [28]. Sweeney (personal communication) has found a copepod alternate host for *A. pinensis* and by a series of careful experiments has

shown that it is quite distinct from the species in *Cx annulirostris*.

A. pinensis differs from *A. indicola* described from *Cx sitiens* in India [32] in spore size, ratio of wide to narrow coils in the polar filament, and in geographical location. Fresh spores of *A. pinensis* measure 5.9 × 3.8 µm cf. 6.7 × 4.0 µm for *A. indicola*. The mean spore length for *A. indicola* (6.7 µm) is outside the range of *A. pinensis* (5.3–6.3 µm, n = 25), while that of *A. pinensis* (5.9 µm) is at the lower end of the range for *A. indicola* (5.7–8.0 µm). Vavra et al. (1984) do not give the standard deviation of their material, making it impossible to test the difference (0.8 µm) between the means statistically, but it is 13 times the standard error of the mean of *A. pinensis* (0.06 µm) indicating that the two means are very different. In *A. indicola* the polar filament has equal numbers of wide and narrow coils (3–4), while in *A. pinensis* there are more narrow (3.5–5) than wide (2–3.5) coils.

The octospores of *Amblyospora* are described as being oval with rounded ends when living and truncate at one or both ends when stained. They have a mucous envelope, a thick exospore and an anisofilar polar filament [10]. The octospores *A. pinensis* meet all these criteria. In *Amblyospora* the episporonal space contains dense granular metabolic products, and chromosomes can be easily seen in dividing sporonts [10]. Both these characters are present in *A. pinensis* with chromosomes being readily seen in both meront and sporont (Figs. 15, 25).

Typical diplokarya have been seen in meronts (Fig. 12). The development of *A. pinensis* in *Cx sitiens* is similar to that of *A. culicis* in *Cx quinquefasciatus*, Say [29]. In the early sporont two nuclei can be distinguished, which are clearly but narrowly separated for much of the common boundary but the separation cannot be traced completely (Fig. 43). This situation recalls the statement of Cossins and Bowler [8] that the apparent diplokaryon in the sporoblast of *Thelohania contejeani* Henneguy was a single nucleus with a deep cleft. In the nuclei of developing sporonts spindle plaques and synaptonemal complexes have been recognised (Fig. 45). The complexes are trilaminar with central and lateral components, similar to those described by Hazard et al. [9] for *Amblyospora* and *Parathelohania*.

Microsporidium lotaensis

The second infection found in *Ae vigilax* was at first considered to be a species of *Nosema* Naegeli but later, stages in a sporogonial cycle were found. In Larsson's [17] key this microsporidian ran down to *Amblyospora* but it should not be referred to that genus until information is available on its biology. For the present it will be included in *Microsporidium*, a genus reserved for unclassified microsporidians. In Larsson's [17] table of genera *M. lotaensis* came closest to the octosporous forms of *Vairimorpha* Pilley and *Amblyospora* with which it shares diplokaryotic meronts and sporonts, uninucleate octospores in a sporophorous vesicle, rosette-like budding of sporont, anisofilar polar filament, and lamellate polaroplast. Its spores are truncate at one end, similar to those of

A. californica, Kellen et Lipa [19] but lack the mucous coat found in most species of *Amblyospora*, and its chromosomes are not easily visible in stained dividing sporonts [10].

In Larsson's [16] table of ultrastructural data for selected Microsporidia *M. lotaensis* again comes closest to the octosporous form of *Vairimorpha*. In both the sporophorous vesicle is type VD with a thin envelope (< 10 nm); the episporontal space contains wide tubules (75 nm) (similar tubular bodies have been figured for *A. keenani* by Hazard and Oldacre [10]); the polaroplast is type I, and the exospore type III but differing in subtype. *Vairimorpha* is type IIIB with a thin exospore (c. 35 nm) and *M. lotaensis* type IIIC with a thicker exospore (90–150 nm).

Pilley [23] established the genus *Vairimorpha* for a microsporidian which was dimorphic, having a *Nosema* type sporogonial cycle at high temperatures (25 °C) and having both a *Nosema* type cycle and an octosporous cycle at low temperatures (20 °C). The two forms of this parasite of Lepidoptera were originally described as separate species, *Nosema necatrix* Kramer, 1965 and *Thelohania diazoma* Kramer, 1965. It is possible that *M. lotaensis* should be placed in the genus *Vairimorpha*. This would be consistent with the abundance of unassociated spores and small numbers of octospores. Our material was obtained in the autumn when temperatures would be declining, favouring an octosporous cycle.

Further study is required to decide the appropriate genus for this species. Information is wanted on two aspects: the effect of temperature on the sporogonial cycle, and whether free spores are produced in the adult female.

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Key words: Microsporidia – *Amblyospora pinensis* – *Duboscqia aediphaga* – *Microsporidium lotaensis* – *Aedes vigilax* – *Culex sitiens*

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