JOURNAL OF INVERTEBRATE PATHOLOGY 62, 137–146 (1993)

Life Cycle of a New Species of *Duboscqia* (Microsporida: Thelohaniidae) Infecting the Mosquito *Anopheles hilli* and an Intermediate Copepod Host, *Apocyclops dengizicus*

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Received May 13, 1992; accepted February 17, 1993

A new species of Microsporida, *Duboscqia dengihilli*, was found infecting larvae of the mosquito *Anopheles hilli* in northern Queensland, Australia. Laboratory experiments showed that binucleate spores formed within infected female mosquitoes were responsible for transovarial transmission to the next generation. Sporogony within the larval fat body was initiated by two diplokarya, one at each end of the cell, which undergo meiosis within a single sporophorous vesicle to form 16 meiospores. These spores are responsible for horizontal transmission to the copepod *Apocyclops dengizicus*. The microsporidium is transmitted back to the mosquito host via uninucleate pyriform spores formed within the copepod host which are infectious to larvae of *A. hilli*. The meronts within larvae infected by horizontal transmission ultimately develop into the binucleate spores within adult females to complete the life cycle. Thus, the development of this microsporidium involves vertical transmission between successive mosquito generations and horizontal transmission between mosquitoes and copepods similar to the life cycles of *Amblyospora* and *Parathelohania*.

KEY WORDS: ***Duboscqia dengihilli;*** **microsporidium;** ***Anopheles hilli;*** **mosquito;** ***Apocyclops dengizicus;*** **copepods; life cycle; intermediate host.**

INTRODUCTION

Surveys of larval mosquito populations were made over a 200-km coastal stretch of north Queensland, Australia between Cairns and Townsville. The objective of this work was to search for microsporidian parasites which may have potential as biocontrol agents of disease vectors. Microsporidian infected larvae of *Culex sitiens, Aedes vigilax,* and *Anopheles hilli* were often found in brackish swamps near the coast. The first mosquito was found to be infected with *Amblyospora indicola,* and subsequent field and laboratory investigations revealed its life cycle in mosquitoes and in its copepod intermediate host (Sweeney *et al.*, 1990). A proportion of larvae in field collections of *A. hilli* from brackish sites near the settlement of Halifax were infected with a previously unidentified species of *Duboscqia*. This genus is characterized by 16 spores within a sporophorous vesicle and has been reported infecting Diptera and Isoptera (Hazard *et al.*, 1981). Infected *A. hilli* larvae, together with associated invertebrate fauna, were transported to our laboratory near Sydney for studies on the development and life cycle of this microsporidium.

MATERIALS AND METHODS

A colony of microsporidian-free *A. hilli* originating from larvae collected at Gove, Northern Territory has been maintained continuously in our laboratory since 1971 and was used for all experiments. This mosquito is autogenous and does not require a blood meal to mature the first egg batch (Sweeney and Russell, 1973). It is readily maintained in 0.03-m^3 cages for mating, blood feeding, and oviposition. Larvae of *A. hilli* with patent fat body infections, together with copepods and other invertebrates, were collected from larval sites near Halifax, northern Queensland (18° 34′S; 146° 18′E) and were airfreighted to Ingleburn at regular intervals for laboratory infection experiments. Copepods were also collected from sites near Taylors Beach (6 km south east of Halifax) and Forrest Beach, approximately 15 km south of Halifax.

Copepod Infection Experiments

Examination of field-collected copepods revealed that there were two cyclopoid species commonly associated with patently infected larvae. One of these copepods was identified as *Apocyclops dengizicus* and the other was an undescribed species of *Apocyclops*. The two species can be distinguished by the morphology of the fifth leg of adult females (D. Morton, pers. commun.). Previous investigations demonstrated that the latter copepod (designated *Apocyclops* sp. nov.) is the intermediate host of *A. indicola* whereas *Apocyclops dengizicus* is not (Sweeney *et al.*, 1990). Field collections containing only one of these two copepod species

0022-2011/93 $4.00

were used for infection experiments by placing ca. 500–2000 adult copepods into plastic trays containing 500 ml of unfiltered water from their collection sites. They were then held in our laboratory at temperatures of 22–26°C. For infection experiments, 2–4 dead or moribund *A. hilli* larvae infected with *Duboscqia* were homogenized to release meiospores and were added to trays of copepods. Samples of 10–40 copepods were removed from the test trays between 3–13 days after exposure to meiospores. They were then smeared, stained with Giemsa, and examined under the light microscope for the presence of microsporidian infections. Trays of copepods, not exposed to meiospores but included in each experiment as a control, were stained and examined for infection at the same time as the test copepods.

Mosquito Infection Experiments

For the initial mosquito infection tests (experiments 1–5) batches of 100–200 first-instar larvae from the *A. hilli* colony were reared in field water containing invertebrates collected from the sites at Halifax and Forrest Beach. Two further experiments (6–7) were performed after the involvement of copepods in the life cycle of this parasite was confirmed. For these latter tests, similar batches of uninfected *A. hilli* larvae from the colony were exposed to *A. dengizicus* infected with *Duboscqia* from the copepod infection experiments described above. Test larvae from all experiments were reared to the pupal stage and placed in cages for adult emergence and mating. The females were provided with blood meals and were then transferred into individual vials of water for oviposition. After the eggs were laid females were smeared, stained with Giemsa, and examined for the presence of *Duboscqia* infection. The larval progeny of females found to be infected were reared in separate sibling batches. Fourth-instar specimens were examined under the dissecting microscope against a black background to identify those with fat body infections. These patently infected larvae were stained with Giemsa and the developmental stages of the microsporidium were examined under the light microscope. The remaining larvae were reared to the pupal and adult stages and then through the subsequent larval generation using the same procedures for identification of infected females and fat-body-infected larvae. For each experiment, control trays of larvae were reared to adulthood using similar procedures to those of the test larvae but without exposure to copepods. The adults which emerged from these trays were Giemsa stained and examined for microsporidian infections.

Electron Microscopy

For light microscope examination, smears of infected specimens were stained with Giemsa according to the methods described by Hazard *et al.* (1981). For electron microscopy, specimens were fixed in 3% glutaraldehyde (v/v) in 0.2 M cacodylate buffer. Specimens were then postfixed in 1% Dalton's chrome osmium (w/v) and uranyl acetate, dehydrated in acetone, embedded in Spurr's resin, sectioned, and examined under a Phillips 300 electron microscope.

RESULTS

Transmission Experiments

Copepods. In one experiment, involving exposure of *A. dengizicus* copepods from Forrest Beach to *A. hilli* larvae with fat-body infections of *Duboscqia,* 7 of 8 test copepods were observed with microsporidian infections which developed to form uninucleate clavate spores. The prevalence of the same microsporidium in test copepods examined from three different experiments, using *A. dengizicus* copepods collected at Halifax were 4/10 (40%), 28/41 (68%), and 4/22 (18%). None of the control copepods examined from the same field collections were infected with microsporidia. In two other experiments, *Apocyclops* sp. nov. copepods from Taylors Beach were exposed to Duboscqia-infected *A. hilli* larvae. None of the test and control copepods examined from these experiments developed microsporidian infections.

Mosquitoes. Mosquitoes from the *A. hilli* colony became experimentally infected with a microsporidium on five separate occasions following exposure of larvae to water and invertebrates from the field collection sites (Table 1, experiments 1–5). Binucleate spores were observed in 10 of 40 females which emerged from trays of larvae exposed to field material from Halifax (9 infected females from four collections) and Forrest Beach (1 infected female from one collection). The progeny of 8 of these females developed patent fat-body infections ranging from 5–100% (average, 65%) of the larvae from each sibling batch. Eggs laid by the other two females did not hatch. The microsporidium within patently infected larvae developed to form groups of 16 uninucleate spores within a sporophorous vesicle. These infections in colony larvae were identical in field-collected *A. hilli* larvae infected with *Duboscqia.* Surviving larvae from infected batches were reared through another generation in an effort to maintain the parasite within the mosquito colony. Only 2 of 34 females (6%) which laid eggs after emerging from patently infected larval batches were infected with binucleate spores. One of these produced 102/103 (99%) patently infected larvae; the nonpatent individual emerged as an uninfected male. The other female produced 17/79 (22%) patent larvae. All of the 19 females which emerged from this larval batch were uninfected; consequently, these attempts to maintain a *Duboscqia-*

TABLE 1

Duboscqia dengihilli Infections in Adults and Larvae of *Anopheles hilli* for Two Generations after Exposure to Either Field-Collected Water and Invertebrates from Sites in Northern Queensland (Experiments 1–5) or to Infected *Apocyclops dengizicus* Copepods (Experiments 6–7)

Experiment no. (source of inoculum)	Parental generation females[a]	F1 Generation larvae[b]	F1 Generation females[c]	F2 Generation larvae[d]
1. (Halifax)	2[e]/11	81/110	1/7	102/103
2. (Forrest)	1/2	5/5	—	—
3. (Halifax)	2[e]/5	13/55	0/12	—
4. (Halifax)	1/7	72/80	0/4	—
5. (Halifax)	4/15	5/102	—	—
		116/144	0/2	—
		25/53	0/7	—
		167/205	1/2	17/79
6. (Infected *Apocyclops dengizicus*)	2[e]/32	7/32	0/11	—
7. (Infected *Apocyclops dengizicus*)	7[e]/11	50/61	0/7	—
		16/18	—	—
		46/141	0/28	—
		46/86	0/14	—
		76/86	1/7	59/59
		0/115	0/26	—

[a] Parental generation females of *A. hilli* which emerged from trays of larvae exposed to either invertebrates and water from collection sites in northern Queensland or to infected *A. dengizicus* copepods. Numbers in columns are No. infected females/total females examined.

[b] Larval progeny (F1 generation) of *A. hilli* females infected in the parental generation. Numbers in columns are No. fat-body-infected larvae/total larvae which hatched from egg batch (laid by corresponding female in previous column).

[c] F1 generation females of *A. hilli* which emerged from trays of larvae infected in the first generation. Numbers in columns are No. infected females/total females which emerged from corresponding batch of first generation larvae.

[d] Larval progeny (F2 generation) of *A. hilli* females infected in the F1 generation. Numbers in columns are No. fat-body-infected larvae/total larvae which hatched from egg batch (laid by corresponding female in previous column).

[e] One egg batch failed to hatch.

infected colony of infected mosquitoes were not successful.

In the first experiment involving exposure of *A. hilli* larvae to infected *A. dengizicus* copepods, 2 of 32 females (6%) and 1 of 19 males (5%) which emerged from the test tray contained binucleate spores (Table 1, experiment 6). One of the infected females produced a batch of 32 larvae of which 7 (6%) developed patent fat-body infections. These infections ultimately developed into 16 meiospores within a sporophorous vesicle and appeared identical to those within the infected larvae which were used for the *A. dengizicus* infection experiments described above. The surviving larvae were reared to adulthood, but none of the 11 females which emerged were infected.

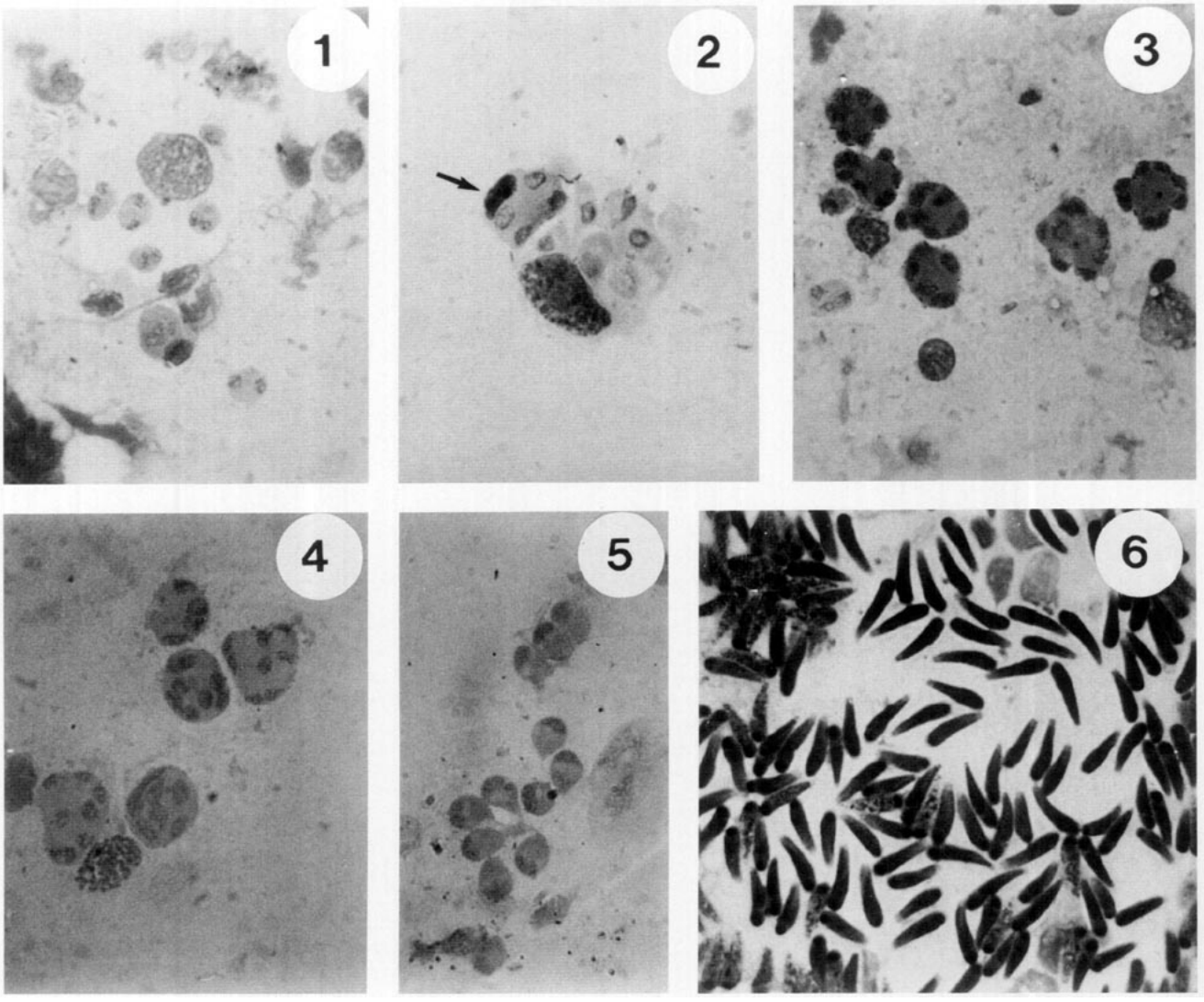
In the final experiment, 7 of 11 females (65%) emerging from the tray of infected copepods developed binucleate spores (Table 1, experiment 7). Six of the infected females produced larvae with fat-body infections ranging from 0–89%. Only 1 of 83 surviving females from these patently infected larval batches developed binucleate spores; all of the 59 larval progeny of this female developed fat-body infections and died in the fourth instar. None of the females which emerged from the control trays of larvae in these experiments were observed with microsporidian infections.

Development in Copepods

In the copepod host, the first stages of this microsporidium were usually seen in Giemsa smears taken 3–5 days after exposure to larval meiospores. These were uninucleate and binucleate stages, some of which were within host cells (Figs. 1, 2). Merogonial multiplication involved repeated nuclear division of these stages resulting in the formation of multinucleate plasmodia (Figs. 3, 4). During sporogony, nuclei migrated to the periphery of the plasmodium; the cytoplasm constricted centrally and cleaved to form uninucleate sporoblasts (Fig. 5). Clavate, slightly curved spores (Fig. 6) were formed 1–2 weeks after exposure. Ultrastructural observations indicate that the spores have a compartmentalized polaroplast and that each is enclosed by a nonpersistent sporophorous vesicle (Fig. 11).

Development in Adult Mosquitoes

Diplokaryotic stages (Fig. 7) were observed within oenocytes of adult female *A. hilli* which were exposed as larvae to infected copepods. These stages developed to form binucleate spores (Fig. 12) responsible for transovarial transmission to the next generation. Binucleate spores were also observed in an adult male

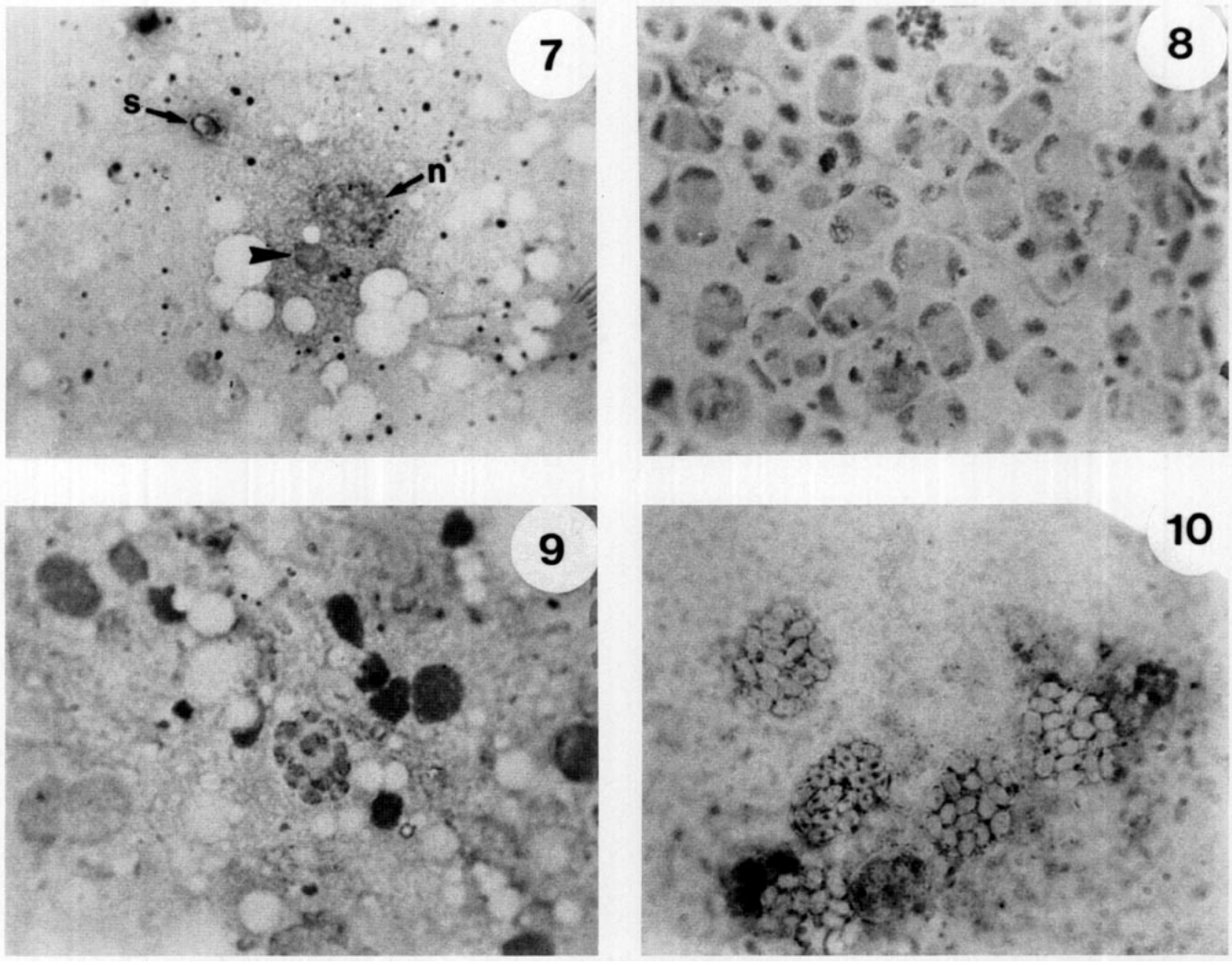


FIGS. 1–6. Light micrographs of *Duboscqia dengihilli* infecting *Apocyclops dengizicus* copepods (all Giemsa stained ×950). Fig. 1, dividing meronts; Figs. 2 and 4, early stages within host cell (host cell nucleus is arrowed); Figs. 3 and 4, multinucleate plasmodia; Fig. 5, cleaving of sporonts; Fig. 6, mature spores.

which emerged from a tray of larvae exposed to infected copepods.

Development in Transovarially Infected Mosquito Larvae

Invasion of the larval fat body with *Duboscqia* initially involves the merogonial multiplication of diplokaryotic stages in first- and second-instar larvae (Figs. 13 and 14) similar to that which occurs with *Amblyospora*. These stages subsequently divide to form two pairs of diplokaryotic nuclei which migrate to opposite poles of the cell immediately prior to the onset of sporogony (Fig. 15). The cytoplasm sometimes appears constricted centrally, but cytokinesis does not occur at this time. The discrete pairs of nuclei then develop through meiotic sequences which appear to be similar to those described for *Amblyospora* by Hazard and Brookbank (1984). Arrangements consistent with meiotic chromosomes at pachytene (Fig. 22), diakinesis (Fig. 24), and metaphase I (Fig. 23) have been observed in Giemsa-stained smears of fat-body-infected larvae. Each pair of nuclei develops separately and undergoes sporogony at each end of the sporont within a single sporophorous vesicle (Figs. 17 and 25). This initially leads to the development of two separate groups of 8 sporoblasts (one group originating from each pair of nuclei) and ultimately results in the formation of 16 uninucleate meiospores enclosed within the sporophorous vesicle (Figs. 10, 21). The exospore layer of the meiospore wall is extended into a small ridge at the posterior end of the mature spore (Fig. 21).



FIGS. 7–10. Light micrographs of *Duboscqia dengihilli* infecting *Anopheles hilli* mosquitoes (Giemsa stained ×900). Fig. 7, diplokaryotic stage (arrowed) within oenocyte (*n* = host cell nucleus) of adult female mosquito and binucleate spore (s); Fig. 8, early sporonts in fat body of infected larva showing discrete pairs of nuclei at opposite ends of cell; Fig. 9, developing sporonts in fat body of larva; Fig. 10, mature meiospores.

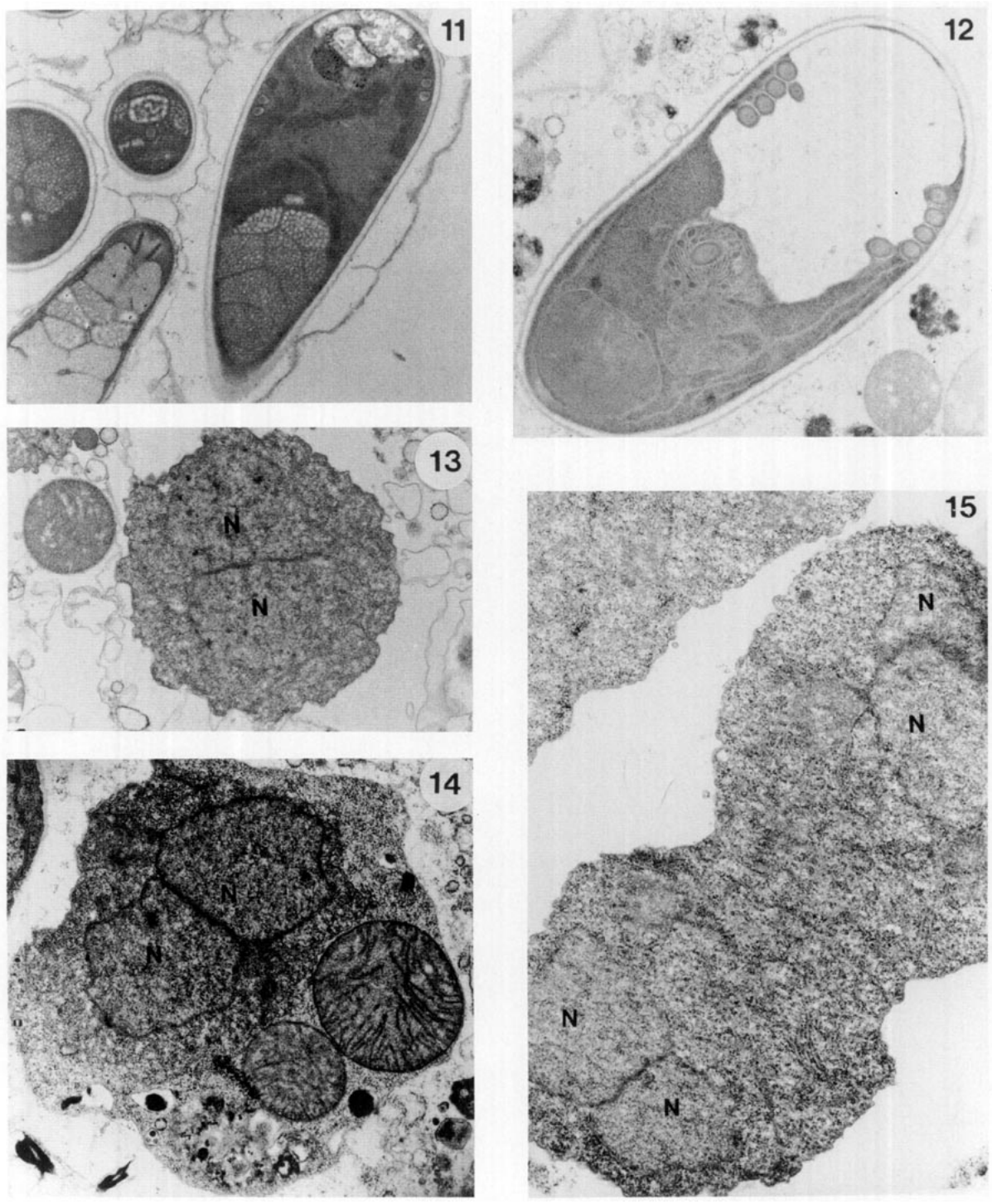
SYSTEMATICS

Duboscqia dengihilli sp. nov.

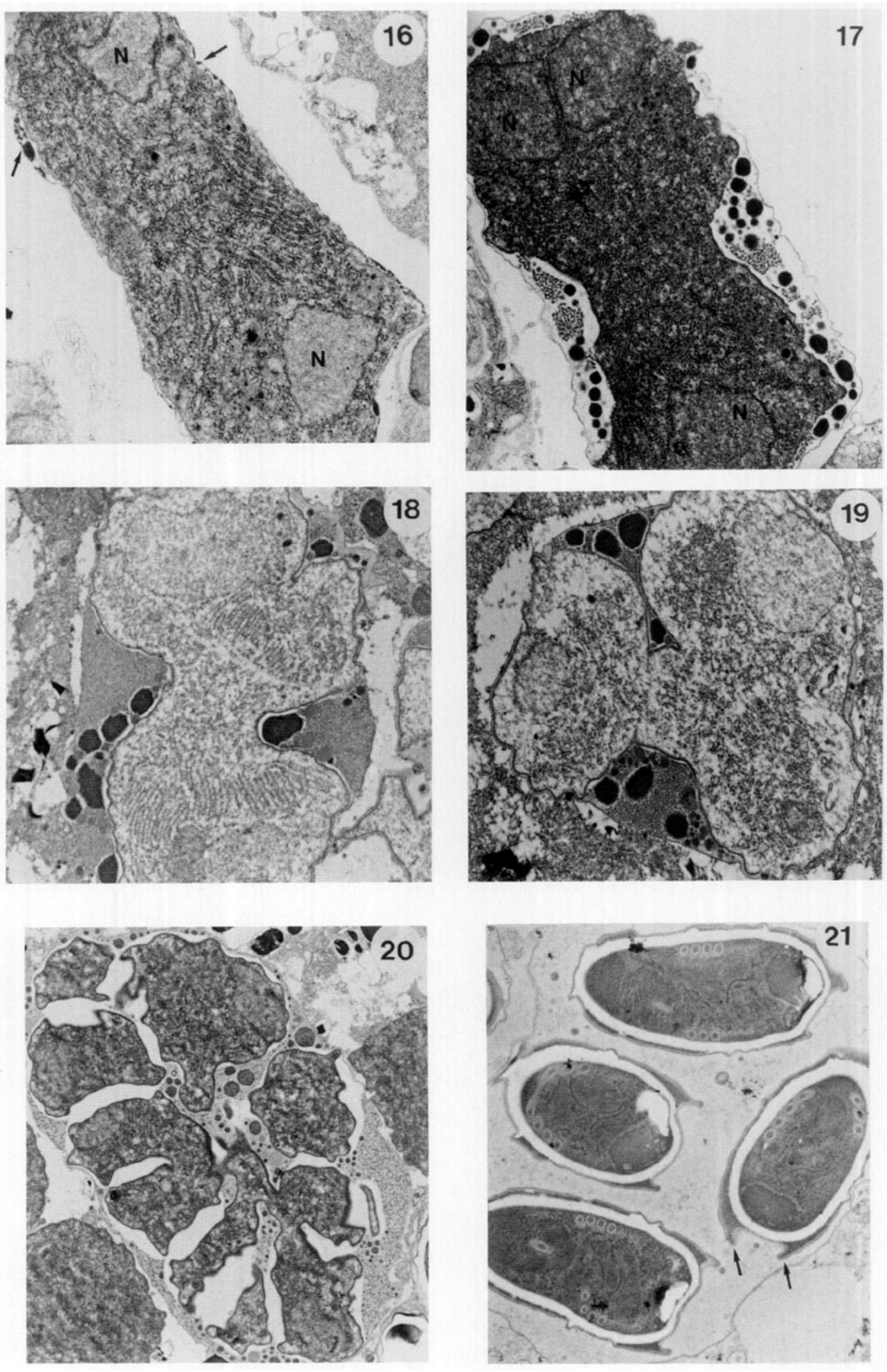
Hosts. *A. hilli* Woodhill and Lee and *A. dengizicus* (Lepeschkin).

Diagnosis. Infections in fat-body tissue of *A. hilli* larvae develop from diplokaryotic meronts which divide to form two pairs of diplokaryotic nuclei, one at each end of the cell. During sporogony, a single sporophorous vesicle encloses the two diplokarya which separately undergo meiosis and form 16 meiospores (8 derived from each diplokaryon). The wall of the meiospore is extended posteriorly to form a ridge similar to that of *Parathelohania* spp. This feature distinguishes the spore from that of *D. aediphaga,* the only other member of this genus which has been reported from a mosquito host. The meiospores are infectious to *A. dengizicus* copepods which develop within host cells to form plasmodia. The cytoplasm of multinucleate sporonts in copepods cleaves to form uninucleate spores, each enclosed by a sporophorous vesicle. Spores formed in copepods are infectious to *A. hilli* larvae. Binucleate spores are formed in adult females following exposure to infected copepods while in the larval stage. These spores are responsible for transovarial transmission to fat-body infections in larval progeny, thereby completing the life cycle.

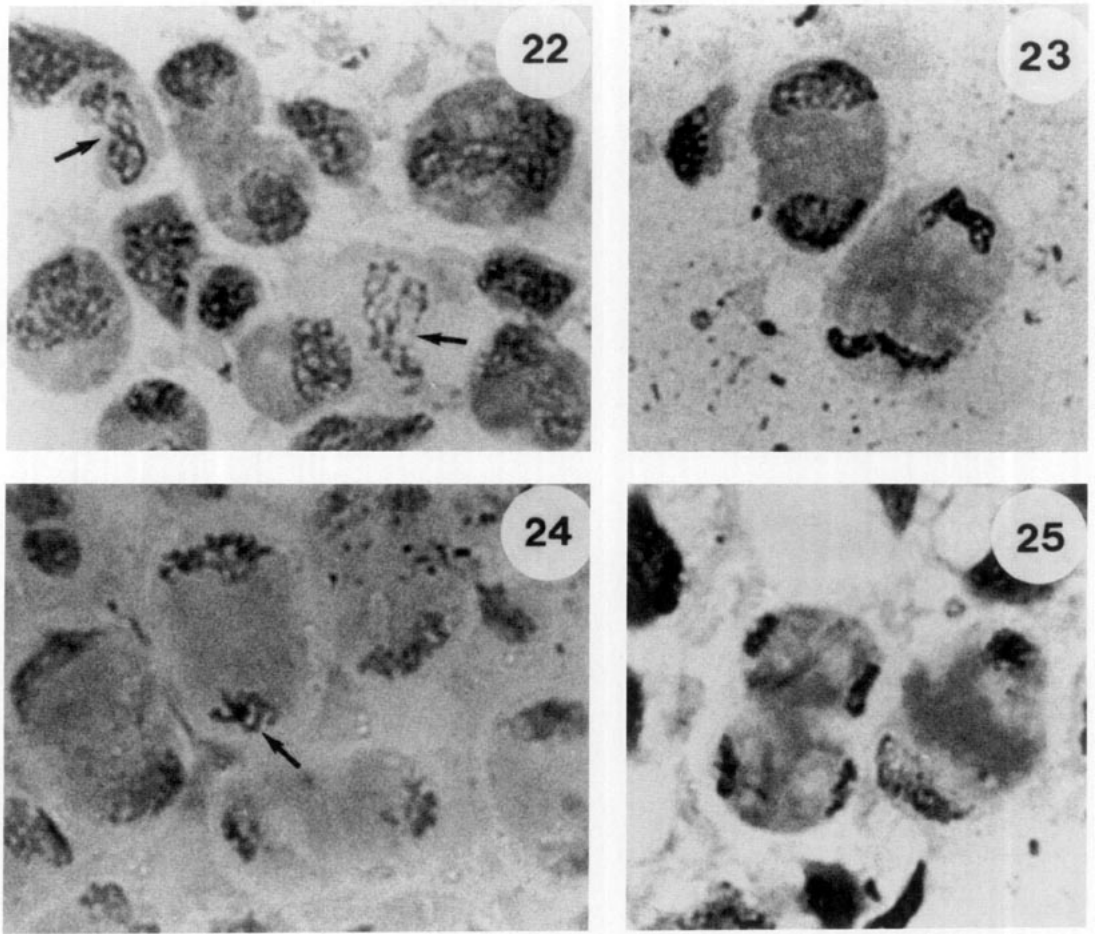
Type slides. Slides will be deposited with the International Protozoan Type Slide Collection, Smithsonian Collection (Washington, DC).



FIGS. 11–15. Electron micrographs of *Duboscqia dengihilli* infecting *Apocyclops dengizicus* copepod (Fig. 11); *Anopheles hilli* adult (Fig. 12) and larvae (Figs. 13–15); *n* = nucleus. Fig. 11, mature uninucleate spore in copepod showing sporophorous vesicle and compartmented polaroplast (×7000); Fig. 12, binucleate spore in adult female mosquito (×15,000); Fig. 13, diplokaryotic meront in larva (×13,500); Fig. 14, diplokaryotic meront within larval host cell (×9800); Fig. 15, quadrinucleate meront showing diplokaryotic nuclei at each end of cell and central constriction of cytoplasm (×8200).



FIGS. 16–21. Electron micrographs of *Duboscqia dengihilli* infecting the fat body of transovarially infected *Anopheles hilli* larvae (Figs. 16–20, ×6300. Fig. 21, ×7600). Fig. 16, early sporont showing nuclei at each end of cell and developing sporophorous vesicle (arrowed); Fig. 17, sporont with centrally constricted cytoplasm and metabolic granules within sporophorous vesicle; Figs. 18–19, dividing sporonts; Fig. 20, cytoplasmic cleavage within sporont prior to formation of 16 sporoblasts; Fig. 21, mature meiospores showing posterior extension of exospore (arrowed).



FIGS. 22–25. Light micrographs of *Duboscqia dengihilli* in fat body of *A. hilli* larvae showing meiotic configurations during sporogony (Giemsa stained ×2000). Fig. 22, chromosomes mingling at pachytene (arrowed); Fig. 23, chromosomes in metaphase at each end of sporont; Fig. 24 appears to show chromosomes at diakinesis (arrowed); Fig. 25, two pairs of binucleate sporonts, one at each end of cell, showing centrally constricted cytoplasm.

Spore Descriptions

Binucleate spores in female A. hilli. Spores are broadly rounded with a thin uniform exospore, a prominent polaroplast, and large posterior vacuole. The polar filament is isofilar with 5–6 coils. Size, 7.4 ± 0.8 μm × 4.6 ± 0.6 μm (stained spores; $n = 20$).

Meiospores in A. hilli larvae. Meiospores are enclosed within a persistent sporophorous vesicle in groups of 16. Spores are rounded with an exospore of variable thickness which is extended posteriorly to form a ridge or keel. The polar filament is anisofilar with 3–4 large coils and 3–5 small coils. Size, 5.0 ± 0.6 μm × 2.8 ± 0.4 μm (stained spores; $n = 20$).

Uninucleate spores in copepods. Spores are clavate and slightly curved, enclosed singly within a thin sporophorous vesicle. Isofilar polar filament with 5–6 coils. Size, 8.8 ± 0.9 μm × 3.0 ± 0.4 μm (stained spores; $n = 20$).

DISCUSSION

The first firm evidence of the involvement of intermediate hosts in the life cycles of microsporidia was the finding in Australia that *Amblyospora dyxenoides* is horizontally transmitted to the mosquito *Culex annulirostris* via spores formed within *Mesocyclops* sp. copepods (Sweeney *et al.*, 1985). Subsequent observations in the United States confirmed that the copepod *Acanthocyclops vernalis* is an intermediate host of *Amblyospora connecticus* infecting the mosquito *Aedes cantator* (Andreadis, 1985) and that *Macrocyclops albidus* is involved in the life cycle of *Amblyospora californica* infecting the mosquito *Culex tarsalis* (Becnel, 1992). *Parathelohania obesa* was horizontally transmitted to *Anopheles quadrimaculatus* by exposing uninfected larvae to water from a field breeding site (Avery, 1989). The source of this infection was not determined but, in later observations, Avery and Undeen (1990) showed that *Parathelohania anophelis* is horizontally trans-

mitted to *A. quadrimaculatus* via spores formed in the copepod *Microcyclops varicans*. The present observations provide another example of this phenomenon in a third genus of heterosporous microsporidia infecting mosquitoes. All three genera are characterized by vertical transmission from infected female mosquitoes to their progeny via binucleate spores, horizontal transmission from mosquitoes to copepods via meiospores, and horizontal transmission from copepods to mosquitoes via uninucleate clavate spores.

The developmental stages and spores of *D. dengihilli* in copepods are very similar, under both the light microscope and the electron microscope, to those described for *Amblyospora* and *Parathelohania*. The diplokaryotic cells within oenocytes and the binucleate spores of this microsporidium in adult mosquitoes (which were exposed to infected copepods as larvae) also resemble those of the other two genera. The development of fat-body infections in transovarially infected larvae is also very similar to those of *Amblyospora* and *Parathelohania*. The stages of *D. dengihilli* in mosquito larvae (infected horizontally via copepods) have not been seen but we presume that this sequence incorporates both gametogenesis and plasmogamy as described by Hazard *et al.* (1985). The only obvious developmental difference between *D. dengihilli* and the other two genera which was noted during the course of this study related to sporogony in the larval fat body. In species of *Amblyospora* and *Parathelohania*, sporogony is initiated by a diplokaryotic cell which develops through meiosis and ultimately forms a sporont with 8 nuclei. With *D. dengihilli* this process commences with two pairs of diplokaryotic nuclei, one at each end of the cell. Each diplokaryon then separately undergoes meiosis and subsequent nuclear divisions result in a sporont with 16 nuclei (8 nuclei from each meiotic configuration). Thus, the 16 spores formed within the sporophorous vesicle of *D. dengihilli* originate from the synchronous development of 2 diplokarya whereas the 8 spores of *Amblyospora* and *Parathelohania* are derived from a single diplokaryon.

The genus *Duboscqia* was established by Perez (1908) for *D. legeri* which forms groups of 16 spores in the termite *Reticulotermes lucifugus*. This microsporidium was also reported in another termite, *Reticulotermes flavipes* by Kudo (1942) who described its developmental cycle within infected adipose tissue. The line drawings of the sporogonic stages in the latter report appear to be consistent with our observations of fat-body infections of *D. dengihilli* involving sporogonial development originating from discrete pairs of nuclei at opposite ends of the sporont. Nevertheless, there would appear to be a need for reexamination of the type species and a possible revision of the genus to accommodate polymorphism.

The only previous record of a member of the genus *Duboscqia* infecting mosquito larvae is *D. aediphaga* which was described by Kettle and Piper (1988) infecting *Aedes vigilax* in Queensland. We have collected this microsporidium infecting *Ae. vigilax* during field surveys and have undertaken laboratory transmission experiments involving a microsporidian free colony of this mosquito and copepods. We were not able to infect *A. dengizicus* copepods with meiospores of *D. aediphaga*. Also, the meiospore of this species does not have the posterior extension evident in *D. dengihilli*. These observations will be reported in a separate communication but they indicate that *D. aediphaga* is not conspecific with *D. dengihilli*.

In all of the *Amblyospora* species which we have studied in Australia, as well as in *Duboscqia* sp. infecting *A. hilli*, mature binucleate spores develop in unfed female mosquitoes and also in males. In some other species of *Amblyospora* infecting North American mosquitoes, sporulation and spore formulation is synchronized with ovarian development (Lord and Hall, 1983; Hall and Washino, 1986) and does not proceed until after the host obtains a blood meal. The formation of mature binucleate spores has not been reported in *Amblyospora*-infected male mosquitoes from North America. However, development of binucleate spores of *P. anophelis* in adult males of *A. quadrimaculatus* has been observed following horizontal infection via copepods (Avery and Undeen, 1990).

Rates of horizontal infection from the intermediate host to mosquitoes are presumably related to the concentrations of uninucleate spores released from copepods into the water containing the test larvae; in our experiments 23% of the females which emerged from trays of larvae exposed to infected copepods harbored microsporidian infections. Infection rates in the larval progeny of infected female *A. hilli* varied considerably in these experiments. In some transovarially infected batches almost all larvae succumbed to patent infections which implies that meiospores can develop in both male and female larvae. In some species of *Amblyospora* and *Parathelohania*, a proportion of larvae from transovarially infected batches develop patent fat-body infections, and other larvae develop benign infections in which the parasite is restricted to diplokaryotic stages within the oenocytes. These benign infections persist to the adult stage and permit the parasite to cycle transovarially for another mosquito generation. The present observations with *D. dengihilli* indicated that benign infections can occur in some transovarially infected individuals. However, this appears to be a rare phenomenon as only 3/128 females (2%) exhibited such infections in the F1 generation. Consequently, almost all transovarially infected larvae in these experiments developed patent infections (and died) and only very few developed benign infections (and survived for another generation). These benignly infected mosquitoes were too few to permit maintenance of the microsporidium within our

laboratory colony of *A. hilli*. Also, we did not have sufficient numbers of *A. dengizicus* to permit continuous *in vivo* maintenance via the copepod and mosquito host. It has been shown that the proportion of transovarially infected larvae acquiring benign infections of *A. dyxenoides* can be influenced by genetic selection (Sweeney *et al.*, 1989) so it is possible that other strains of *D. dengihilli* may have different transovarial transmission attributes.

ACKNOWLEDGMENTS

This paper is published with the approval of the Director General of Army Health Services. The research received financial support from the Australian National Health and Medical Research Council, and from the UNDP/WHO/World Bank Special Programme for Research and Training in Tropical Diseases. We are grateful to Jimmy Becnel, of USDA-ARS, Medical and Veterinary Entomological Research Laboratory, Gainesville, Florida, for critical review of the manuscript. We wish to thank Lorraine Van der Lubbe, Electron Microscope Unit, University of Sydney, for assistance with electron microscopy and David Morton, Department of Zoology, Monash University, for identifying copepods.

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