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Population Biology

Experimental transmission of a microsporidian pathogen from mosquitoes to an alternate copepod host

(*Amblyospora/Aedes cantator/Acanthocyclops vernalis*/ultrastructure)

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ABSTRACT **Meiospores of a microsporidian parasite *Amblyospora* sp. (Protozoa: Microspora) from larval *Aedes cantator* mosquitoes were directly infectious to an alternate copepod host, *Acanthocyclops vernalis* (Arthropoda: Crustacea). Infections ranged from 6.7% to 60.0% in laboratory tests when meiospores and copepods were maintained together for 10–30 days in filtered water from the breeding site or in a balanced salt solution. Pathogen development takes place within host adipose tissue and is fatal to the copepod. The entire developmental sequence of this microsporidian in the copepod is unikaryotic and there is no ultrastructural evidence of a sexual cycle or a restoration of the diploid condition in the alternate host. Single uninucleated spores similar to those previously described for the genus *Pyrotheca* are formed. Results demonstrate that haploid meiospores of *Amblyospora* from mosquitoes have the function of transmitting the pathogen to another host and that members of this genus are polymorphic and have at least three distinct developmental cycles, each producing a different spore.**

Although microsporidia (subkingdom Protozoa, phylum Microspora) of the family Amblyosporidae are one of the most prevalent and widely distributed groups of parasites that infect natural populations of mosquitoes (1, 2), their life cycles and methods of transmission are only partially understood. All members are transovarially transmitted and have two developmental cycles, one usually but not always in each host sex. Each cycle produces a different spore: (*i*) a thin-walled binucleated one that infects the ovaries of adult females and is responsible for transovarial transmission and (*ii*) a thick-walled uninucleated one, termed meiospore, that infects fat body tissue of larvae and kills the host but is not infectious to mosquitoes (3–8).

Horizontal transmission of these pathogens is believed to occur in most if not all mosquito hosts (4–10) but has been observed in only one species, *Aedes stimulans* (Walker), in nature (8). In that study, infections were acquired by larvae during the early stages of development in the field, but the source of infection was not determined.

It has long been suspected (4, 5, 7–10) that meiospores of these microsporidia, which abound in larval hosts but do not infect mosquitoes, might be infectious to another host and subsequently develop into an infective stage that was transmissible to mosquitoes. Successful transmission of a microsporidian pathogen from mosquitoes to another host has recently been achieved by Sweeney *et al.* (11), who report that meiospores of an *Amblyospora* species from an Australian mosquito, *Culex annulirostris* Skuse are infectious to a copepod (phylum Arthropoda, class Crustacea) host and that another spore is formed in this host that in turn infects larval mosquitoes.

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This study reports the results of transmission trials with an *Amblyospora* species from a North American salt-marsh mosquito, *Aedes cantator* (Coquillett), and an indigenous copepod, *Acanthocyclops vernalis* (Fisher, 1853) Kiefer, 1927, and further describes the complete life history and ultrastructure of this microsporidium in the copepod host. The life cycle and field epizootiology of this *Amblyospora* species in the mosquito have been extensively described in earlier studies (7, 10)

MATERIALS AND METHODS

Transmission Tests. The *A. vernalis* copepods used in all experiments were obtained during October 1984 from a coastal salt marsh in Guilford, CT, that served as a major breeding site for *A. cantator*. Copepods were collected on several occasions from an isolated semipermanent pool where large numbers of *A. cantator* larvae were concurrently developing. There was an ongoing epizootic of *Amblyospora* in the larval population and fresh meiospores, procured from these larvae, were the source of inoculum.

All transmission tests were conducted at 22°C in white enamel pans (18 × 29 × 4.5 cm) containing 500 ml of either field-collected water from the pool, filtered through Whatman no. 5 paper to remove all particles down to 2.5 μm, or a balanced salt solution (12). Approximately 100 copepods that had been rinsed in distilled water were placed in each pan along with 10 moribund fourth-instar larvae of *A. cantator* that were heavily infected with *Amblyospora* meiospores. Controls consisted of pans of each solution to which an equal number of copepods but no meiospores had been added. Finely ground Tetramin fish food was added to each pan to help sustain copepod development.

Copepods were held for up to 30 days in each solution and examined for infection at various intervals. Diagnosis of infection was based on the presence of vegetative stages or spores as observed in Giemsa-stained smears of live individual copepods. The prevalence of infection at each time interval was determined from the examination of at least 23 live specimens from each sample pan. A number of copepods were also smeared, stained, and examined immediately following each collection to ascertain whether any natural infection was present within each sample.

Life Cycle Studies. Pathogen development in *A. vernalis* was characterized by examining Giemsa-stained smears of both lightly and heavily infected individuals in which the pathogen exhibited various degrees of development. This was complemented by ultrastructural studies of copepods with similar levels of infection. For the ultrastructural studies, whole copepods were fixed overnight at 4°C in 2.5% (wt/vol) glutaraldehyde containing 0.1% (wt/vol) $CaCl_2$ and 1% (wt/vol) sucrose, and buffered with 0.1 M sodium cacodylate (pH 7.4). Specimens were postfixed at room temperature with 1% (wt/vol) OsO_4 in the same buffer, dehydrated through an ethanol series, and stained *en bloc* overnight at 4°C with 0.5% (wt/vol) uranyl acetate in 70%

Table 1. Prevalence of *Amblyospora* infections in *A. vernalis* that were maintained in filtered site water or balanced salt solutions to which fresh meiospores from *A. cantator* were added

Date collected	No. days held	Filtered site water				Balanced salt solution			
		With meiospores		Without meiospores		With meiospores		Without meiospores	
		No.	% infected	No.	% infected	No.	% infected	No.	% infected
Oct. 4	0	—	—	25	0	—	—	25	0
	15	27	14.8	27	0	25	60.0	26	0
	20	28	32.1	26	0	30	6.7	23	0
	25	54	51.9	51	0	—	—	24	0
Oct. 25	0	—	—	25	0	—	—	25	0
	10	24	25.0	25	0	26	53.8	25	0
	30	—	—	25	0	—	—	25	0

(vol/vol) ethanol. Whole copepods were embedded in an LX-112/Araldite mixture after 2 days of infiltration. Sections were poststained with 5% (wt/vol) methanolic uranyl acetate, followed by Reynolds lead citrate, and examined in a Zeiss EM-9 electron microscope at an accelerating voltage of 60 kV.

RESULTS

Transmission Tests. Copepod infections with *Amblyospora* were readily achieved in all transmission trials in which meiospores were added to the rearing water (Table 1). This occurred regardless of whether the tests were conducted in filtered water from the breeding site or in a balanced salt solution. At the same time, not a single infected copepod was collected from the field or found in any of the control pans that were maintained under identical conditions but to which no meiospores had been added. These observations clearly indicated the source of infection in the copepods was *Amblyospora* meiospores that were added to the medium.

Infection rates in copepods that were initially collected on Oct. 4 and maintained in filtered site water ranged from 14.8% to 51.9% and showed a steady increase in prevalence throughout the 25-day exposure period. Examination of individual copepods after 15 days of exposure revealed the presence of vegetative stages only. Spores were first detected in specimens that were examined after 20 days and predominated after 25 days. High infection rates were obtained with shorter exposure periods in copepods that were held in salt solutions (60% vs. 14.8% after 15 days). However, this also led to early mortality of infected individuals and by day 25, dead copepods filled with spores were seen but no live copepods could be found. Similar results were obtained with copepods that were collected and exposed to meiospores on Oct. 25.

Pathogen Life Cycle. The earliest developmental stages observed in copepods with light infections were small (8- to 10-μm) uninucleated meronts (Figs. 1*A* and 2*A*). These underwent repeated nuclear divisions and formed large multinucleated plasmodia (up to 30 μm) that possessed as many as 12 unikaryotic nuclei (Figs. 1 *B–F* and 2*B*).

Meronts were located within host adipose tissue and they appeared irregular in shape at the ultrastructural level (Figs. 2 *A* and *B*). They were characteristically bound by a thin plasmalemma that was in direct contact with the host cell cytoplasm and they contained a dense homogeneous cytoplasm that was also rich in ribosomes.

Cytoplasmic cleavage of merogonial plasmodia was occasionally observed in Giemsa-stained smears (Fig. 1*F*). This gave rise to a number of uninucleated cells (sporonts) that were ovoid and possessed a large nucleus at one pole (Fig. 1*G*). These stages were distinguished from meronts at the ultrastructural level by their thickened plasmalemma and more diffuse cytoplasm (Fig. 2*C*).

Sporonts appeared to undergo a short sporogonial sequence during which binucleated and quadrinucleated stages with centrally constricted cytoplasms were formed by synchronous nuclear divisions (Figs. 1 *H–J* and 2 *D* and *E*). These stages had a very distinctive budlike appearance and their cleavage gave rise to additional uninucleated stages that presumably underwent sporulation.

Early sporoblasts (Figs. 1*K* and 2*F*) were observed to secrete a sporophorous vesicle. This appeared as a separate double unit membrane exterior to the plasmalemma (Fig. 2*F*).

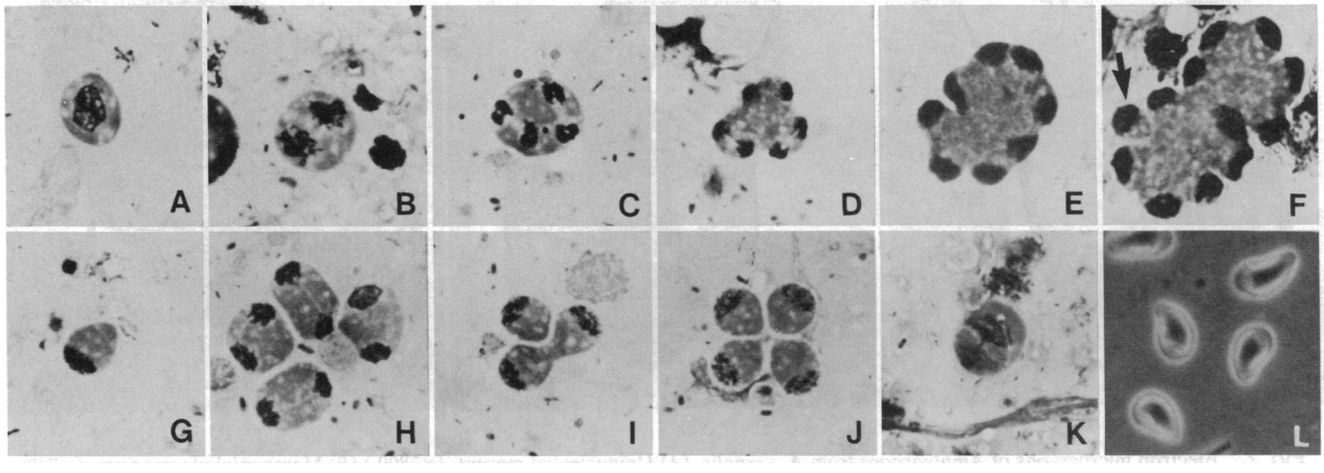

FIG. 1. Stages of *Amblyospora* development from *A. vernalis*. (All Giemsa stained except *L*; all ×1000.) (*A*) Uninucleated meront. (*B*) Binucleated meront. (*C* and *D*) Dividing meronts. (*E* and *F*) Merogonial plasmodia (arrow indicates cleaved sporont). (*G*) Uninucleated sporont. (*H–J*) Dividing sporonts. (*K*) Sporoblast. (*L*) Fresh live mature spores.

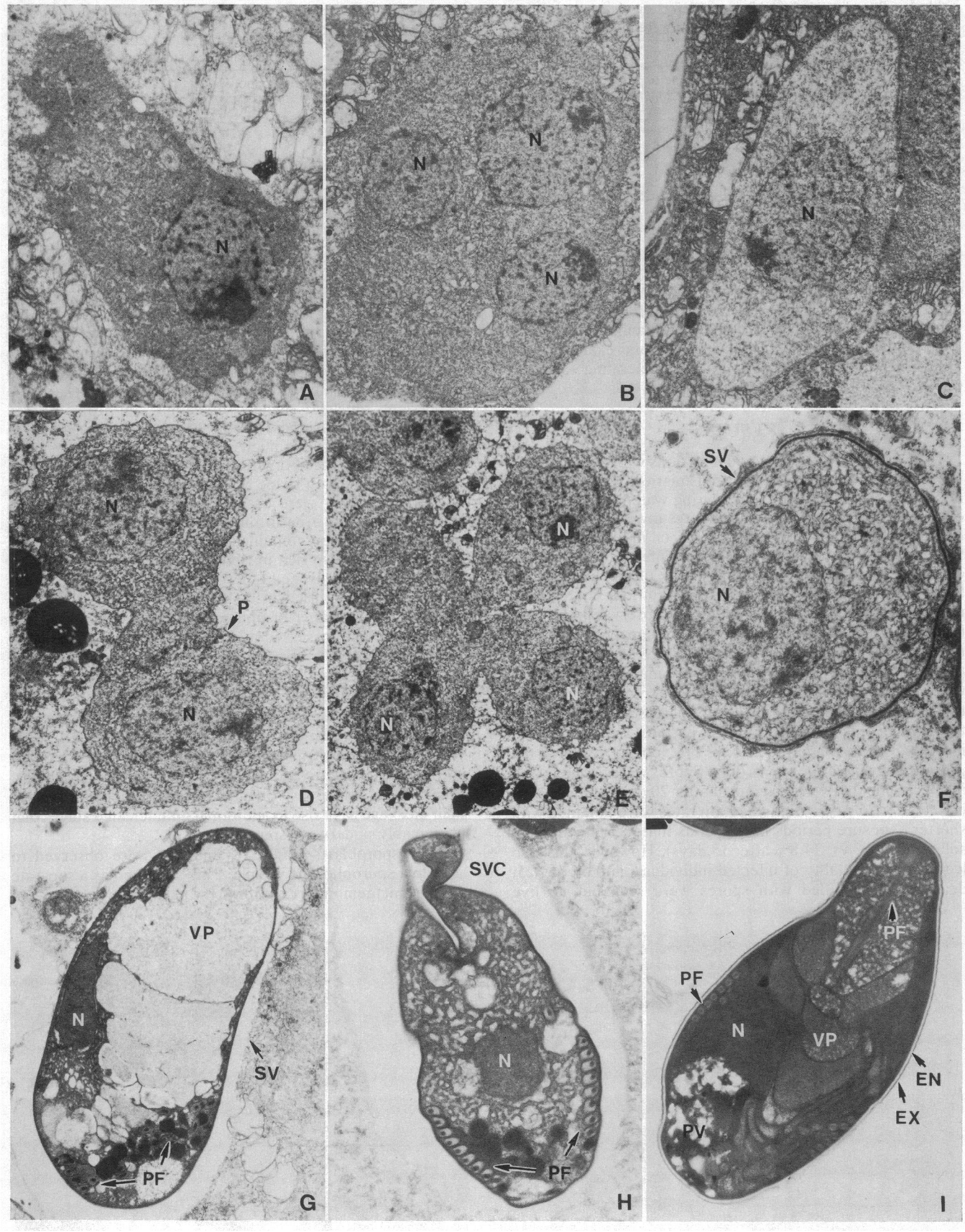

FIG. 2. Electron micrographs of *Amblyospora* from *A. vernalis*. (*A*) Uninucleated meront. (×7800.) (*B*) Merogonial plasmodium. (×7400.) (*C*) Uninucleated sporont. (×8300.) (*D*) Dividing binucleated sporont. (×7300.) (*E*) Quadrinucleated sporont. (×4800.) (*F*) Early sporoblast. (×13,600.) (*G*) Sporoblast. (×8200.) (*H*) Sporoblast (×12,700.) (*I*) Spore. (×13,600.) EN, endospore; EX, exospore; N, nucleus; P, plasmalemma; PF, polar filament; PV, posterior vacuole; SV, sporophorous vesicle; SVC, sporophorous vesicle cavity; VP, vesicular polaroplast.

These sporoblasts also possessed a very thick plasmalemma and a highly vacuolated cytoplasm rich in endoplasmic reticulum.

During sporogenesis, sporoblasts appeared to elongate and the membrane of the sporophorous vesicle became fully detached from the plasmalemma, creating a distinct vesicular cavity that completely enclosed the sporoblast (Fig. 2 *G* and *H*). The most prominent organelles in these stages were the large vesicular polaroplast and the developing polar filament.

Mature spores (Figs. 1*L* and 2*I*) were pyriform with a slightly curved and pointed anterior end. They were 8–10 μm × 5–6 μm (fresh) and were uninucleate. They possessed a large vesicular polaroplast that occupied the interior two-thirds of the sporoplasm. The polar filament was of the isofilar type (of uniform diameter) and consisted of 11–12 coils. The posterior vacuole was large and the exospore and endospore were both relatively thin walled.

DISCUSSION

The results obtained in these transmission tests show that meiospores of *Amblyospora* sp. from larval mosquitoes of *A. cantator* are directly infectious to an alternate copepod host, *A. vernalis*. This finding is highly significant because it confirms the findings of Sweeney *et al.* (11) and further establishes that meiospores of *Amblyospora*, which abound in larval mosquitoes worldwide but are not infectious to their original host, do have a function and are not aberrant. It also demonstrates that species of *Amblyospora*, and undoubtedly others within the Amblyosporidae, are polymorphic and have at least three separate and distinct developmental pathways, each of which produces a morphologically different spore. This degree of polymorphism as well as the successful biological transmission of an insect microsporidian to a member of another arthropod class are not presently known for any other members of the Microspora.

Transmission of *Amblyospora* to copepods does not appear to require any special conditioning of meiospores, and infections can be readily achieved in the laboratory both in filtered water from the breeding site and in a balanced salt solution. The conditions under which infections take place in nature, however, are unclear, since no infected copepods were collected from the field. This was surprising because large numbers of infected larvae, which provided the source of inoculum in the laboratory, were present within the pool throughout the entire sampling period. Lack of infection in field-collected copepods may have been due to an insufficient quantity of meiospores in the microhabitat of the host or the presence of one or more physical factors that inhibited the transmission process. An elucidation of the conditions and factors that influence transmission in nature will require further comprehensive study.

Pathogen development takes place within host adipose tissue and infections are ultimately fatal to the copepod. The entire developmental sequence is unikaryotic and there is no ultrastructural evidence of karyogamy or gametogony. This would seem to indicate that the uninucleated spores produced in the copepod are also haploid and that there is no sexual cycle or restoration of the diploid condition in this host.

Development is initiated by uninucleated meronts that undergo repeated nuclear divisions and form multinucleated plasmodia with up to 12 nuclei. These subsequently cleave and give rise to uninucleated sporonts that undergo further division to produce two or four uninucleated sporoblasts. Sporulation is pansporoblastic (occurs within a sporophorous vesicle) and results in the formation of individually enclosed uninucleated spores.

The development of this pathogen in *A. vernalis* and structural features of the spore are similar in many regards to those described for the genus *Pyrotheca* Hesse, 1935 (13). This is a poorly defined and little known group consisting of only four species, all of which have been described from copepods and other microcrustaceans (14–17). The results obtained in this study raise serious questions about the validity of this group, since it is now highly probable that members of *Pyrotheca* may actually represent intermediate stages of *Amblyospora* and thus be synonomous. The correct taxonomic placement of these polymorphic microsporidia that develop in different hosts but have been previously described will probably require a case-by-case study of each species in question in order to establish its conspecificity with existing species. In doing so, one may also want to consider which host is definitive. In this instance, for example, since meiosis, karyogamy, and diploid development occur within the mosquito (4, 5, 7, 18) and development within the copepod appears to be haploid, the mosquito should be considered the definitive host and thus these microsporidia should be retained within the genus *Amblyospora*. However, *Pyrotheca* Hesse, 1935, was established prior to *Amblyospora* Hazard and Oldacre, 1975, and therefore may take precedence according to the International Code of Zoological Nomenclature. In any event, the existence of polymorphism and development in alternate hosts of these and undoubtedly other microsporidia will necessitate a reevaluation of current concepts of microsporidian taxonomy and a complete redefinition of both the family and genus.

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