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Amblyospora khaliulini (Microsporidia: Amblyosporidae): Investigations on its life cycle and ecology in *Aedes communis* (Diptera: Culicidae) and *Acanthocyclops vernalis* (Copepoda: Cyclopidae) with redescription of the species

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ABSTRACT

A multi-year study was conducted to examine the natural ecology of the microsporidium *Amblyospora khaliulini* and more fully characterize parasite development and histopathology in all stages of its primary mosquito host, *Aedes communis* and intermediate copepod host, *Acanthocyclops vernalis* with redescription of the species. *A. khaliulini* exhibits polymorphic development, produces three morphologically and functionally distinct spores, and is both horizontally and vertically transmitted. Development in *A. vernalis* is restricted to females, occurs within the ovaries and results in death of the host. Development is haplophasic with division by binary and multiple fission producing rosette-shaped sporogonial plasmodia and conical uninucleate spores that are orally infectious to *Ae. communis* larvae. Both sexes are equally susceptible and infections are confined to testes in males and ovaries in females. Initial stages of development include uninucleate schizonts that undergo karyokinesis forming diplokaryotic meronts that divide repeatedly by binary fission. Sporogony occurs in both host sexes, but sporogenesis does not progress normally in adult males and elliptical, thin walled binucleate spores that function in vertical transmission of the microsporidium via infection of the ovaries and eggs are formed in adult females only. Development of vertically acquired infections in larval *Ae. communis* hosts occurs within fat body tissue, leads to the production of meiospores in male hosts only and results in death during the 4th larval stadium. Initial development is characterized by merogonial multiplication of diplokarya by synchronous binary division producing additional diplokarya. The cessation of merogony and the onset of sporogony are characterized by the simultaneous secretion of a sporophorous vesicle and meiotic division of diplokarya resulting in the formation of octonucleate sporonts that undergo cytokinesis and sporogenesis to form eight uninucleate, broadly ovoid meiospores enclosed within a sporophorous vesicle. The natural prevalence of patent vertically acquired fat body infections in field populations of *Ae. communis* ranged from 1.6% to 3.6%. Yearly infection rates in *A. vernalis* copepods ranged from 57.1% to 15.0%. Prevalence rates of horizontally acquired infections in emerging adult *Ae. communis* ranged from 69.0% to 11.9% in males and 50.0% to 16.4% in females.

1. Introduction

Amblyospora khaliulini Hazard and Oldacre, 1975 is a little known microsporidian parasite of the univoltine, boreal mosquito, *Aedes communis* (DeGeer) (Hazard and Oldacre, 1975). First recognized in 1920 as a species of *Thelohania* from a few patently infected larval mosquitoes with “white cysts” collected in Germany (Noller, 1920), it was similarly reported from late stage larval specimens collected in the Czech Republic (*Thelohania opacita*) (Weiser, 1947); the former U.S.S.R (*Thelohania opacita* var. *mariensis*) (Khaliulin and Ivanov, 1971); Manitoba, Canada (*Thelohania* sp.) (Welch, 1960); Alaska (*Thelohania* sp.) (Chapman et al., 1973) and Massachusetts (holotype) (Hazard and Oldacre, 1975) in the United States. Welch (1960) remarked on its gross pathology noting that the parasite typically killed the larval host by infecting fat body tissue and preventing host pupation. He further reported that it was present in a high percentage of forest pools (up to 86%) in Churchill, Manitoba and was responsible for a reduction of 3–11 percent of the larval population. Rudimentary observations on the sporogonic sequence producing “octospores” (now known as meiospores) and spore size were also documented from the larval host, but no attempt was made to identify the source of infection and no other aspects of the life cycle were revealed.

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Hazard and Oldacre (1975) subsequently revised the *Thelohania* and established a new genus, *Amblyospora* to include many mosquito-parasitic species with dimorphic development and oval truncated "octospores". *Amblyospora khaliulini* was accordingly assigned and redescribed. However, this was based solely on morphological characteristics and ultrastructure of the spore produced in infected larvae. No further details on parasite development, morphology or pathology in adult hosts, transmission mechanisms or features of the life cycle were identified, nor the involvement of the intermediate copepod host which had yet to be discovered.

While conducting a survey for microsporidia infecting natural populations of mosquitoes inhabiting forested wetlands in the northeastern United States, *A. khaliulini* was recovered from an isolated population of *Ae. communis* inhabiting a semi-permanent, vernal pool in northwestern Connecticut, USA. Accordingly, we initiated a multi-year study to examine the natural ecology of *A. khaliulini* and more fully characterize parasite development and histopathology in all stages of both the mosquito and intermediate copepod hosts by light and electron microscopy with full redescription of the species.

2. Materials and methods

2.1. Study site and field ecology

The study site was an evergreen forest, dominated by eastern hemlock (*Tsuga canadensis*) and eastern white pine (*Pinus strobus*) located in Barkhamsted, Litchfield County, CT, USA (41° 57′ 48″N, 72° 53′ 94″W). The aquatic habitat was a well-defined leaf-lined, vernal pool that was typically dry by June or early July (Fig. 1.). The pool supported populations of two univoltine mosquitoes, *Ae. communis* and *Aedes excrucians* (Walker) and the cyclopoid copepod, *Acanthocyclops vernalis* (Fischer).

Field studies were conducted in 1999, 2000, 2001 and 2005 for the purpose of assessing the natural prevalence of *A. khaliulini* infection in *Ae. communis* and its intermediate host, *A. vernalis* throughout their respective stages of development within the aquatic habitat. Sampling was conducted weekly from the onset of larval mosquito hatch (late March to early April) until pupation (mid to late May). Immature mosquitoes and copepods were collected from the pool with a standard 350 ml mosquito dipper and immediately transported to the laboratory for examination. Mosquitoes were identified using keys and descriptions from Darsie and Ward (1981) and Means (1979). Copepods were identified using keys and descriptions of Yeatman (1959) and Pennak (1989). Confirmation on the identity and conspecificity of *A. khaliulini* from both hosts was corroborated from SSU rDNA sequences obtained from mature spores procured from naturally infected *Ae. communis* larvae and adult female *A. vernalis* (GenBank/EMBL database Accession Nos. AY090045, AY090046, AY090047) (Vossbrinck et al., 2004).

Fig. 1. Vernal pool study site as seen in early April with early melting along perimeter.

The weekly prevalence rate of *A. khaliulini* infection in both mosquito and copepod populations was determined from microscopic (1000x) examination of Giemsa-stained smears (10% solution, pH 6.8) of a minimum of 50 whole mosquito larvae, and up to 50 whole adult female copepods collected on each sample date. Smears were air dried, fixed in 100% methanol (5 min), and stained with a 15% (v/v) modified Giemsa stain solution (pH 7.4) (20 min) (Sigma-Aldrich Accustain® Giemsa Stain, Modified, St. Louis, MO). Larval mosquito development was typically uniform, but in instances where mixed larval instars were collected, equal numbers of each were examined in an effort to obtain a prevalence rate that was representative of the entire population. Only female copepods were examined as prior investigations with other species of *Amblyospora* (Andreadis, 1988a) and *Hyalinocysta* (Andreadis and Vossbrinck, 2002) had shown males to be refractory to infection owing to the site of parasite development in ovarian tissue.

In order to assess infection prevalence in the emerging adult mosquito population, field collected pupae were individually isolated in 30-ml plastic containers, held at room temperature and similarly examined for infection 1–2 days after emergence. Overall prevalence rates were based on examination of an equal number of males and females (up to 50 each). In all instances, individual copepods and mosquitoes were scored as infected if any developmental stage (vegetative or spore) was observed.

2.2. Life cycle studies: Light microscopy

General characterization of microsporidian development in both the mosquito and copepod hosts was made from microscopic examination (1000x) of Giemsa-stained smears of infected tissues obtained from live mosquito larvae, emerging adults and female copepods collected from the field, and from larval *Ae. communis* infected in the laboratory transmission studies (see Section 2.4). Tissue specificity was determined from histological examination of paraffin-embedded whole larval, adult male and female stages of *Ae. communis* and plastic embedded *A. vernalis*. Paraffin sections were stained with iron hematoxylin and eosin Y. Measurements of mature spores were calculated from examination of whole wet-mount preparations of live spores (n = 50) with differential interference optics in a Zeiss Axioplan 2 Digital imaging system (1000x).

2.3. Life cycle studies: Ultrastructure

The comparative ultrastructure of microsporidian development was ascertained from examination of adult female *A. vernalis* and larval and adult male and female stages of *Ae. communis*. Infected tissues were fixed in a 2.5% (v/v) glutaraldehyde solution containing 0.1% (w/v) $CaCl_2$ and 1% (w/v) sucrose buffered in 100 mM Na cacodylate (pH 7.3) overnight at room temperature and postfixed in 1% (w/v) OsO_4 in the same buffer and temperature. Fixed specimens were dehydrated through a graded ethanol and acetone series and embedded in a LX-112/Araldite (Ladd Research Industries, Williston, VT) mixture. Thin sections (60–100 nm) were stained with 5% (w/v) uranyl acetate in 50% (v/v) methanol followed by Reynold's lead citrate, and examined in a Zeiss EM 10C electron microscope at an accelerating voltage of 80 kV.

2.4. Transmission studies

Horizontal transmission studies were conducted in the laboratory to (1) qualitatively assess the oral infectivity of spores of *A. khaliulini* procured from field collected *A. vernalis*, and (2) corroborate the source and type of infection observed in larval populations of *Ae. communis* in