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Epizootiology of *Hyalinocysta chapmani* (Microsporidia: Thelohaniidae) infections in field populations of *Culiseta melanura* (Diptera: Culicidae) and *Orthocyclops modestus* (Copepoda: Cyclopidae): a three-year investigation

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Abstract

The epizootiology, transmission dynamics and survival strategies employed by the microsporidium *Hyalinocysta chapmani* were examined in field populations of its primary mosquito host, *Culiseta melanura* and its intermediate copepod host, *Orthocyclops modestus* over a three-year period in an aquatic subterranean habitat. *H. chapmani* was enzootic and was maintained in a continuous cycle of horizontal transmission between each host. There were three distinct periods during the summer and fall when developing mosquito larvae acquired infections; each was preceded by or coincident with the detection of infected copepods. Results were corroborated in laboratory bioassays, wherein transmission was achieved in mosquito larvae that were reared in water and sediment samples taken from the site during the same time periods. The highest infection rates, ranging from 60% to 48%, were repeatedly observed during the first six weeks of larval development. These were coincident with the most sustained collections of infected copepods obtained during the year and highest levels of infection achieved in the laboratory transmission studies. The high prevalence rates of lethal infection observed in larval populations of *C. melanura* at this site are among the highest recorded for any mosquito-parasitic microsporidium and clearly suggest that *H. chapmani* is an important natural enemy of *C. melanura*. *H. chapmani* appears to overwinter in diapausing mosquito larvae but may also persist in copepods. The absence of vertical transmission in the life cycle of *H. chapmani* and the sole reliance on horizontal transmission via an intermediate host are unique survival strategies not seen among other mosquito-parasitic microsporidia. The epizootiological data suggest that this transmission strategy is a function of the biological attributes of the hosts and the comparatively stable environment in which they inhabit. The subterranean habitat is inundated with water throughout the year; copepods are omnipresent and *C. melanura* has overlapping broods. The spatial and temporal overlap of both hosts affords abundant opportunity for continuous horizontal transmission and increases the likelihood that *H. chapmani* will find a target host. It is hypothesized that natural selection has favored the production of meiospores in female host mosquitoes rather than congenital transfer of infection to progeny via ovarian infection as a strategy for achieving greater transmission success.

Keywords: *Hyalinocysta chapmani*; Microsporidia; *Culiseta melanura*; Mosquito; *Orthocyclops modestus*; Copepod; Epizootiology; Horizontal transmission

1. Introduction

Hyalinocysta chapmani Hazard and Oldacre, 1975 is a polymorphic microsporidian parasite of the mosquito *Culiseta melanura* Coquillett and the copepod *Orthocyclops modestus* (Herrick). It represents one of the four microsporidian genera (*Amblyospora*, *Duboscqia*, and *Parathelohania*) that infect mosquitoes (primary host) and utilize copepods as intermediate hosts. Its life cycle has recently been elucidated (Andreadis and Vossbrinck, 2002) and is described below. Infections arise in *C. melanura* larvae following the oral ingestion of uninu-

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cleate spores previously formed in infected copepods. Spores germinate within the lumen of the midgut and directly invade fat body tissue where all development occurs. The microsporidium undergoes two phases of asexual reproduction (schizogony and merogony), followed by meiosis and a prolonged sporulation sequence that culminates in the production of tens of thousands of haploid meiospores. These are released into the aquatic habitat with the death of infected larvae, which usually succumb during the fourth stadium. Some infected individuals occasionally survive to adulthood due to a less intense infection, but there is no further parasite development that leads to either maternal- or paternal-mediated vertical transmission. *H. chapmani* is horizontally transmitted to *O. modestus* via oral ingestion of meiospores. Infections become established within ovarian tissue of female hosts and large numbers of uninucleate spores are produced. The ovaries become grossly distended, egg development is inhibited and infections eventually become systemic. This results in death of the copepod and subsequent dispersal of spores into the aquatic environment where they are ingested by mosquito larvae to initiate a new cycle.

Hyalinocysta chapmani is unique among all mosquito-parasitic microsporidia that have thus far been described. It lacks a developmental sequence leading to transovarial transmission in the mosquito host, and relies exclusively on horizontal transmission via a copepod host to complete its life cycle. Analysis of molecular phylogeny data (Andreadis and Vossbrinck, 2002) suggests that transovarial transmission has been secondarily lost in *H. chapmani*, as it occurs in all other closely related genera (*Amblyospora*, *Edhazardia*, *Culicosporella Culicospora*, and *Parathelohania*). It has also been hypothesized that the life cycle and transmission strategies employed by this microsporidium are an adaptation to local host ecology.

The objectives of the present investigation were to examine the natural epizootiology and host-parasite-host transmission dynamics of *H. chapmani* in field populations of its primary mosquito host, *C. melanura* and its intermediate copepod host, *O. modestus* over a three-year period, and to further examine the transmission and survival strategies employed by this microsporidium.

2. Materials and methods

2.1. Host biology

Culiseta melanura is a widespread mosquito that is distributed throughout the eastern and central United States (Darsie and Ward, 1981). It is a multivoltine species that has two generations a year in the northeastern United States (Mahmood and Crans, 1998) and is among the most dominant mosquitoes found in fresh water swamps and sphagnum bogs in Connecticut (Andreadis et al., 1994). Females feed primarily on birds (Nasci and Edman, 1981) and are active from June to October (Andreadis et al., 2001). Eggs are laid in water in cryptic subterranean habitats, and larvae develop in holes beneath mats of sphagnum and in deep shaded cavities around the roots of upturned trees (Pierson and Morris, 1982) where water temperatures remain below 20 °C most of the summer (Mahmood and Crans, 1998). Larval development is exceptionally slow. Under controlled conditions egg hatch to adult emergence takes 8 mo at 10 °C, 3 mo at 16 °C, and 1 mo at 22 °C (Mahmood and Crans, 1998). The species is unusual in that it overwinters in the larval stage. The overwintering generation of larvae emerge as adults in the spring (June) and produce a summer generation that emerges in the fall (August and September). Eggs laid by the late summer/fall brood of adults produce larvae that make up the overwintering generation that emerge the following spring (Mahmood and Crans, 1998).

Orthocyclops modestus is a small (0.8–1.25 mm) copepod species that is widely distributed throughout the United States and southern Canada (Yeatman, 1959). According to Reid (2001), it is only occasionally reported; however, because it typically inhabits small, ephemeral, vernal pools, and shallow wells that are not often sampled. It has been reported to be numerically dominant in shallow (<0.5 m deep) moist soil impoundments in open areas of river floodplains, and in beaver ponds during the winter and spring in the southeastern United States (Duffy and LaBar, 1994). It survives dry periods in a dormant resting stage (Burno et al., 2001), but very little is known about its life history.

2.2. Site description and collection methods

The study was conducted in a densely wooded fresh water swamp (sphagnum bog) located at Mohawk State Forest in Cornwall, Litchfield County, CT, USA (41°48′45″N, 73°17′41″W). The sample site was an aquatic subterranean habitat located beneath an open cavity (0.5 m diameter) at the root base of an overturned tree (Fig. 1) that bordered (<10 m) a large permanent pond (Mohawk Pond). The habitat contained water throughout the year. Sampling was conducted weekly from March to November for three-consecutive years (1995–1997). The site was inaccessible due to ice cover from December to February. Larval mosquitoes and copepods were collected with a standard "mosquito dipper" and a modified bilge pump (Becksin Industrial Products) (Woodrow and Howard, 1994) as indicated by the level of water in the cavity. Samples were immediately transported to the laboratory for examination. Copepods were identified using diagnostic keys and descriptions of Yeatman (1959), Pennak (1989), and

Fig. 1. Aquatic subterranean sampling habitat for *Culiseta melanura* and *Orthocyclops modestus* at root base of an overturned tree at Mohawk State Forest in Cornwall, CT.

Dussart and Defaye (1995). Associated species in addition to *O. modestus* included: *Acanthocyclops vernalis* (Fischer), *Acanthocyclops venustoides* Coker, *Ectocyclops phaleratus* (Koch), *Eucyclops agilis* (Koch), *Macrocyclops albidus* (Jurine), *Macrocyclops fuscus* (Jurine), and *Tropocyclops prasinus* (Fischer). Mosquitoes were identified using keys and descriptions of Darsie and Ward (1981), and Means (1979, 1987). Associated species in addition to *C. melanura* included: *Aedes cinereus* Meigen, *Culiseta morsitans* (Theobald), and *Ochlerotatus canadensis* (Theobald).

2.3. Assay procedures

The natural prevalence of *H. chapmani* within the larval population of *C. melanura* was quantified from microscopic (1000×) examination of Giemsa-stained smears (10% solution, pH 6.8) of a minimum of 50 larvae that were collected each week. The comparative abundance of each larval instar was additionally recorded. Where mixed instars were present, equal numbers of each instar were examined in an effort to obtain a prevalence rate of infection that reflected the larval population. Since all infections resulted in death of the larva and meiospore production, there was no need to differentiate the level or type of infection in each host larva. Larvae were scored as infected if any developmental stage (vegetative or spore) was observed.

Whole adult *O. modestus* copepods (mostly female) were similarly examined and scored for infection with *H. chapmani*. However, because of the cryptic nature of the species and difficulty in obtaining large consistent sample sizes (ave. = 7.8 copepods/sample, range 1–26), no attempt was made to calculate a weekly prevalence rate of infection. Records were only kept on the presence or absence of infection within the sampled copepod population. Naupli and copopodite stages were not examined.

2.4. Laboratory transmission studies

Laboratory transmission studies were undertaken weekly to corroborate observations on the prevalence of *H. chapmani* infection in field-collected *C. melanura* larvae and to more clearly define the natural transmission dynamics between the two hosts. In these experiments, 50 s instar *C. melanura* larvae, obtained from an infection-free laboratory colony, were reared in 100 × 80-mm culture dishes containing 100 ml of unfiltered water and sediment collected from the sample site. A small amount of an aqueous suspension of liver powder and Brewer's yeast was provided for food. Larvae were reared at 25 °C, under a 16:8 LD photoperiod for 14 days, following which they were smeared on microscope slides, stained with Giemsa, and examined microscopically for infection as described previously. Controls consisted of an equal number of larvae that were similarly reared in distilled water. Bioassays were conducted in 1995 and 1996 but not in 1997 due to the unanticipated loss of the *C. melanura* colony.

3. Results

3.1. Field study 1995

In 1995, *H. chapmani* was immediately found in overwintering *C. melanura* larvae with the onset of ice melt in mid-March (Fig. 2). Larvae with similar infection rates were repeatedly collected thereafter until pupation and the initiation of adult emergence in early June. The overall prevalence rate of infection in the overwintering larval population was 8.1% ($n = 627$).

First and second instar larvae of the summer generation of were detected in mid-June and 15% were initially found to be infected. Infection rates increased steadily over the next 6 weeks to a high of 60% in early August. A steady gradual decline in the prevalence of infection was observed through August. This was followed by a second, albeit lower rise in the prevalence of infection in mid-September to 30%, and subsequent decline to zero in mid-October. A final increase to 20% was observed in early November. The overall prevalence rate of infection in the summer larval population of *C. melanura* was 26.4% ($n = 988$).

The first indication of *O. modestus* copepods within the larval habitat was in early June and several were found infected. With exception of one sample week in late July, infected copepods were repeatedly collected

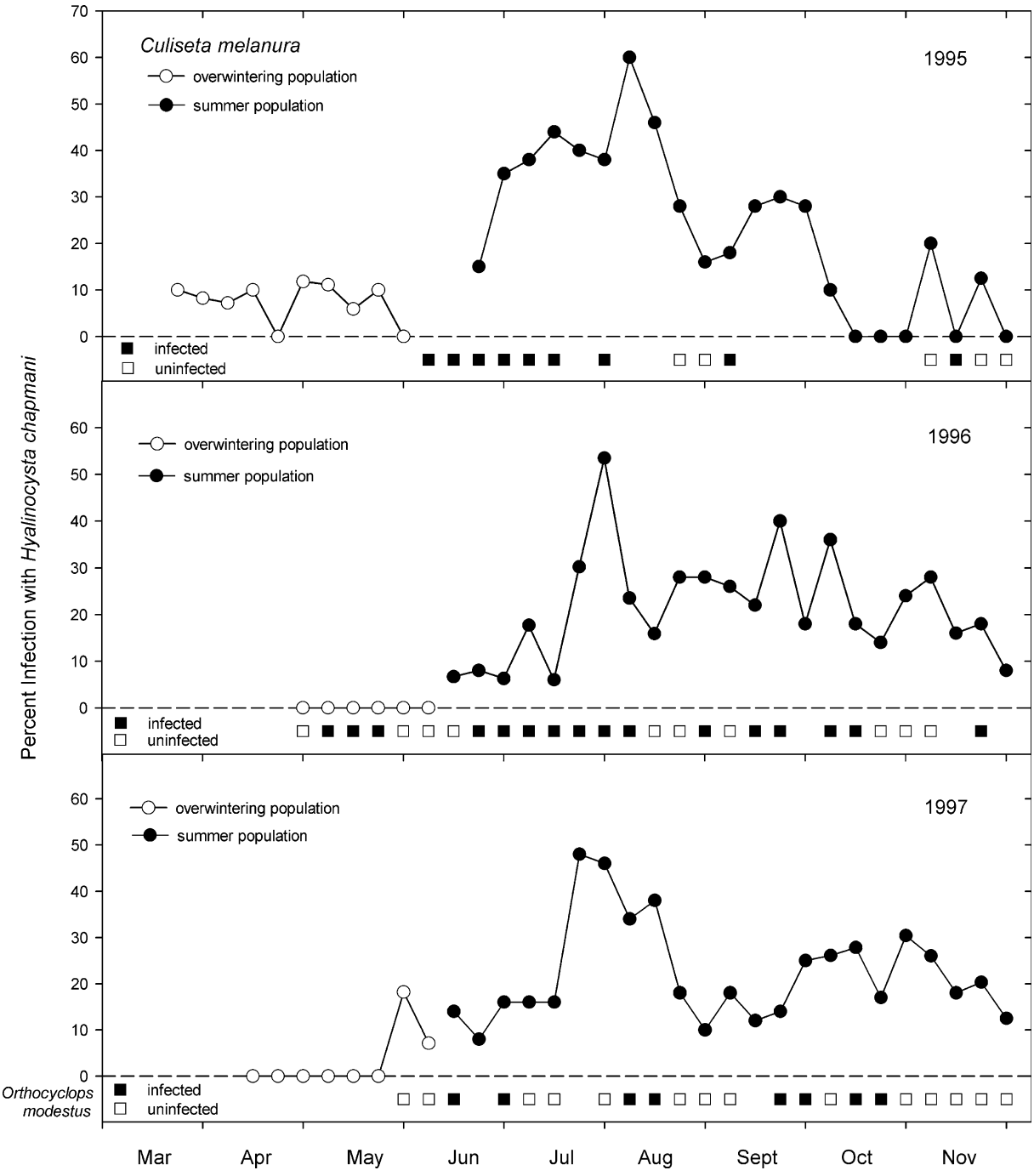

Fig. 2. Weekly prevalence of *Hyalinocysta chapmani* infections in larval populations of *Culiseta melanura* and adult populations of *Orthocyclops modestus* at Mohawk State Forest in Cornwall, CT, 1995–1997.

over the next 7 weeks until the end of July. This was coincident with the rise in the prevalence of infection in the larval mosquito population. The overall prevalence rate of infection in this cohort of copepods was 28.8% ($n = 59$). Thereafter, copepods were irregularly collected and infected individuals were found on only 2 occasions (early September and November). The overall prevalence rate of infection in the copepod population was 23.2% ($n = 95$).

3.2. Field study 1996

Ice melt occurred much later in the spring of 1996 than in 1995. The first water samples could not obtained until early May; no *H. chapmani* infections were detected in any overwintering *C. melanura* larvae that were examined through early June ($n = 90$) (Fig. 2). Infected *O. modestus* copepods were detected on 3 of 6 occasions during the same sampling period (infection

rate = 12.1%, $n = 58$). As in 1995, a steady gradual increase in the prevalence of infection was observed in the summer generation of *C. melanura* larvae during June and July; the highest level of infection recorded was 53.5%. The rise in the prevalence of infection in *C. melanura* was concomitant with the detection of *H. chapmani* in *O. modestus* copepods as was similarly documented in 1995 (infection rate = 21.4%, $n = 117$). Infection rates in *C. melanura* declined thereafter and this was followed by 2 subsequent increases in mid-late September (40%) and early November (28%) before declining to a low of 8% just prior to freezing of the habitat. The overall prevalence of infection in the summer population of *C. melanura* was 21.2% ($n = 1203$). Unlike 1995, *O. modestus* copepods were present throughout most of the late summer and fall months, and infected individuals were collected on 6 additional occasions. The overall prevalence of infection in the copepod population was 19.1% ($n = 236$).

3.3. Field study 1997

The first collections of overwintering larval mosquitoes in 1997 were obtained in mid-April, but no individuals infected with *H. chapmani* were recovered until June (Fig. 2). This was coincident with the first detection of *O. modestus* copepods, but none were infected. The overall prevalence of infection in the overwintering mosquito population was 3.2% ($n = 95$), less than one-half of what was recorded in 1995 but more than 1996. The seasonal patterns and prevalences of *H. chapmani* infection observed in the summer population of *C. melanura* were very similar to those observed in the two preceding years. Infections gradually increased to a peak prevalence rate of 48% in mid-July. This was followed by a gradual decline through August and two additional increases in mid-October (27.8%) and November (30.4%). The latter two increases were preceded by the detection of infected copepods. The overall prevalence rate of infection in the summer population of *C. melanura* was 22.6% ($n = 1081$). Copepods were recovered less frequently in 1997 than in 1996 and on only 8 occasions were infections with *H. chapmani* recorded. The overall prevalence of infection in the copepod population was 9.1% ($n = 232$), the lowest of the three sample years.

3.4. Laboratory transmission studies

In 1995, transmission was achieved in larvae reared in water samples collected from June to September (Table 1). The highest prevalence rates of infection were obtained in June (32.0%) and July (45.0%). No infections were obtained in any larvae reared in water samples taken from the site in the early spring (April–May) or late fall (October–November). Similar, but slightly different results were obtained in 1996. No transmission was achieved with water samples collected in the spring and early summer (April–June) and late fall (November). The highest prevalence rates of infection were again obtained in July (20.0%), but they were less than half of what was obtained in July of 1995. Comparable prevalence rates to 1995 were achieved in August (8.0%) and September (14.0%), and a small level of transmission was obtained with water samples collected in October (2.5%).

3.5. Additional microsporidia from other hosts

Three different microsporidia were additionally isolated from the copepods, *A. vernalis, M. albidus*, and *M. fuscus*. Although the identities of these microsporidia have yet to be definitively determined, rDNA sequencing revealed they were each distinct species and not *H. chapmani* (Vossbrinck, per. comm.). No microsporidian infections were detected in co-habiting mosquitoes, *Ae. cinereus*, *Cs. morsitans*, and *Oc. canadensis.*

4. Discussion

Within this isolated population of *C. melanura*, *H. chapmani* was enzootic and was maintained in a continuous cycle of horizontal transmission throughout the summer and fall (June–November) via its intermediate host, *O. modestus*. Although some variation occured from year to year, there appeared to be three distinct periods when developing mosquito larvae of the summer generation acquired infections; mid-June to August, September to early October, and early November. With a few exceptions, each of these transmission events was either preceded by or coincident with the detection of

Table 1
Results of laboratory bioassays showing percent infection with *Hyalinocycta chapmani* in *Culiseta melanura* larvae reared in field-collected water samples 1995–1996

		Month of collection of water samples							
		April	May	June	July	August	September	October	November
1995	No. exposed	200	250	150	200	275	100	200	200
	% infection	0	0	32.0	45.0	5.5	10.0	0	0
1996	No. exposed	100	250	150	300	200	250	200	200
	% infection	0	0	0	20.0	8.0	14.0	2.5	0

infected copepods in the habitat, thus supporting this summation. This interpretation of the field observations was also corroborated by the laboratory bioassays, wherein transmission was achieved in mosquito larvae that were reared in water and sediment samples taken from the site during the same time periods, thus confirming an infectious level of spore inoculum in the habitat.

Peak infection rates, ranging from 60 to 48% were repeatedly attained during the first six weeks of larval development in each of the three years. This was interpreted as the maximum period of amplification of infection within the larval population. The increase in prevalence was consistent with the slow rate of larval mosquito development at this time, and absence of any parasite-induced mortality until just prior to pupation (Andreadis and Vossbrinck, 2002). The high infection rates were also coincident with the most sustained collections of infected copepods obtained during the year and highest levels of infection achieved in the laboratory transmission studies. The subsequent decline in the prevalence of infection in the larval population was attributed to the death of infected fourth instar larvae and the recruitment of newly hatched larvae into the population.

The prevalence rates observed in *C. melanura* were equivalent to the prevalences of horizontally transmitted infections of *Amblyospora connecticus* reported in *Ochlerotatus cantator* populations in coastal salt marshes following oral ingestion of spores produced in its intermediate copepod host, *A. vernalis*. These infections, which do not kill the host but rather result in ovarian infection and transovarial transmission, typically average 40% and may be as high as 60% in first generation larvae that develop over an 8-week period in the spring when copepod populations are similarly abundant (Andreadis, 1990). The high levels of horizontal transmission observed with *H. chapmani* in the present study; however, contrasted sharply with the comparatively low prevalence rates of (copepod-induced) horizontally transmitted infections reported with *Amblyospora albifasciati* in a multivoltine mosquito, *Ochlerotatus albifasciatus* (6.4–20%) (Miceli et al., 2001), and with *Amblyospora stimuli* in a univoltine mosquito, *Ochlerotatus stimulans* (0.1–8.7%) (Andreadis, 1999). It is significant that unlike *H. chapmani*, all three of these *Amblyospora* species rely heavily on maternal-mediated transovarial transmission for survival, and their hosts develop in more ephemeral habitats that are subject to periodic flooding and drying. In *A. stimuli*, the low rates of horizontal transmission of infection to mosquito larvae have been attributed to a paucity of copepods in the aquatic habitat (Andreadis, 1999). Overall, the high prevalence rates of lethal infection observed in larval populations of *C. melanura* at this site are among the highest recorded for any mosquito-parasitic microsporidium. Unfortunately, because of the cryptic nature of the larval habitat and continuous recruitment of neonate larvae into the population, we were not able to directly measure larval density and the subsequent impact of this microsporidium on the population. However, the high prevalence rates clearly suggest that *H. chapmani* is an important natural enemy of *C. melanura*.

The recovery of *H. chapmani* from overwintered *C. melanura* larvae collected in the early spring of 1995 and 1997 indicates that this microsporidium likely overwinters in diapausing mosquito larvae. This is consistent with the biology of the mosquito host. The detection of infection in copepod populations in May of 1996 also suggests that *H. chapmani* may additionally overwinter in copepods. However, while *O. modestus* is reported to survive dry periods in a dormant resting stage (Burno et al., 2001), it is unknown how or in what stage (nauplius, copepodid, adult) the species overwinters in this type of habitat. It has been established (Andreadis, 1990) that another closely related microsporidium, *A. connecticus* effectively overwinters in late stage copepodid and adult *A. vernalis,* but additional studies on the biology of *O. modestus* are needed to determine if *O. modestus* survives the winter in the same way.

Although no attempt was made to evaluate the survival or viability of free spores in the external environment, the lack of transmission with water samples collected in April and May indicates that *H. chapmani* is unlikely to overwinter outside of the host. This conclusion is also supported by an earlier study (Andreadis, 1991) in which meiospores of *A. connecticus* exhibited a significant loss in viability to copepods after storage in water for 5 months at 4 °C. In summary, it appears that *H. chapmani,* like other mosquito-parasitic microsporidia (Andreadis, 1990; Lucarrotti and Andreadis, 1995), survives in one of two living hosts through much of its life cycle rather that in the external environment.

The absence of vertical transmission in the life cycle of *H. chapmani* and the reliance on horizontal transmission via an intermediate host are unique survival strategies not seen among other mosquito-parasitic microsporidia. Vertical transmission is found in all other closely related genera (*Amblyospora*, *Edhazardia*, *Culicosporella*, *Culicospora*, and *Parathelohania*), and is generally viewed to be the single most important adaptation for survival that has evolved within these mosquito-parasitic species (Lucarotti and Andreadis, 1995). Comparative phylogenetic analysis of the rDNA sequences of representative species within the aforementioned genera (Andreadis and Vossbrinck, 2002), further imply that transovarial transmission and the developmental sequence leading to ovarian infection have been secondarily lost in *H. chapmani*. If the loss of transovarial transmission is adaptive rather than accidental, then this evolutionary event must be examined in the context of how it confers greater reproductive fitness

and transmission success to *H. chapmani*. The epizootiological data obtained herein suggest that this is a function of the biological attributes of the hosts and the environment in which they inhabit. The aquatic subterranean habitat that supports each host is inundated with water throughout the year and is very stable. Copepods are omnipresent and *C. melanura* has overlapping broods. Susceptible mosquito larvae in various stages of development can be found in intimate association with these copepods throughout much of the year. The spatial and temporal overlap of both hosts thus affords abundant opportunity for continuous horizontal transmission and increases the likelihood that *H. chapmani* will find a target host. Natural selection would therefore favor any developmental pathway that increases the parasites' ability to infect another host and achieve greater transmission success, in this instance, maximizing the production of large numbers of infective spores in each host. This presumes that the production of meiospores in female mosquitoes will result in greater overall transmission success than the congenital transfer of infection to progeny via ovarian infection. This interpretation supports the hypotheses of Sweeney et al. (1989) and Hurst (1991, 1993) who have argued that where vertical transmission is poor and adequate copepod populations are available, the production of lethal meiospores in female as well as male mosquitoes can be an optimal strategy. This host-parasite relationship, which can be influenced by genetic selection in the laboratory (Sweeney et al., 1989), has been observed among certain species of *Amblyospora* that infect multivoltine mosquitoes that inhabit more permanent bodies of water (Kellen et al., 1965; Anderson, 1968; Sweeney et al., 1988, 1989; Andreadis, 1990). Further epizootiological and phylogenetic investigations which examine the life history strategies of each parasite and host(s) are clearly needed to more fully appraise these theories.

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