EFFECT OF *PER OS EDHAZARDIA AEDIS* (MICROSPORIDA: AMBLYOSPORIDAE) INFECTION ON *AEDES AEGYPTI* MORTALITY AND BODY SIZE

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ABSTRACT. Infection with *Edhazardia aedis* uninucleate spores had less effect on *Aedes aegypti* larval mortality and adult body size than did larval diet. Larval mortality averaged 60–81% in starved larvae and 2–16% in well-fed larvae. No significant amounts of larval mortality could consistently be attributed to exposure to the parasite at dosages of 1.5×10^3 or 1.5×10^5 spores/ml. Infection rates in adults surviving exposure to the parasites as larvae ranged from 30 to 59%. Infected adults had significantly smaller body sizes than uninfected adults or controls. Storage of spores in water reduced infectivity gradually over the course of 36 h. By 48 h, the spores were not able to infect mosquito larvae. Spore infectivity was eliminated by drying.

INTRODUCTION

Microsporidia have been reported in over 100 mosquito species throughout the world (Hazard and Chapman 1977, Castillo 1980). However, only 3 species, *Nosema algerae*, *Amblyospora connecticus* and *Amblyspora dyxenoides* have been investigated extensively as biocontrol agents for mosquitoes (Anthony et al. 1978, Andreadis 1988, Sweeney et al. 1988). The lack of research with microsporidia is partially due to the lack of knowledge concerning the life cycle of the parasites.

The subject of this paper, *Edhazardia aedis* (Kudo) Becnel et al. 1989, is a microsporidian parasite of *Aedes aegypti* (Linn.) originally reported from Puerto Rico (Kudo 1930) and later from Thailand (Hembree 1979). Chapman et al. (1987) described a very similar parasite from *Aedes albopictus* (Skuse) in Malaysia, though it was not verified as *E. aedis*. Much is known about the life cycle of *E. aedis* (Hembree and Ryan 1982, Hazard et al. 1985, Becnel et al. 1989). The dose response of *Ae. aegypti* to this species was examined by Hembree (1982). Hazard (1985) classified *E. aedis* as having a Type IV life cycle. Microsporidia of this type are considered to have the most potential as biocontrol agents (Hazard 1985).

The life cycle of *E. aedis* in *Ae. aegypti* is complex with some important variations (Becnel et al. 1989), but the typical life cycle occurs as follows: Mosquito larvae are infected by ingesting uninucleate spores in the larval habitat. If 1st or 2nd instars are exposed to high concentrations of these spores, larval mortality is very high (Hembree 1982). However, if the spore concentration in the habitat is moderate, infected larvae develop to the adult stage. The parasite invades the ovaries of the infected adult mosquito, and is passed transovarially via a binucleate spore. The transovarially infected larvae develop to the 4th instar, at which time the parasite causes larval mortality. Larval death results in the liberation of infective uninucleate spores into the larval habitat. These spores, if ingested by other mosquito larvae, will initiate a new infection. The maintenance of the parasite in the mosquito population by transovarial transmission, the potential to cause significant larval mortality and the potential to reduce mosquito fitness (Hembree 1982) make this parasite an attractive biocontrol agent (Hazard 1985).

We examined the effect of *per os* infection with *E. aedis* spores and larval diet on *Ae. aegypti* larval survival and adult body size in the laboratory. We also determined the duration of viability of the parasite spores when in aqueous suspension and when dried.

MATERIALS AND METHODS

Edhazardia aedis spore suspensions were prepared by hatching transovarially infected *Ae. aegypti* eggs and rearing larvae to the 4th instar. When spores were numerous, the larvae were concentrated and macerated with a small amount of distilled water in a tissue grinder. The mosquito homogenate was filtered through a single layer of tissue paper to remove large particles, and the concentration of spores in the filtrate was determined with a hemocytometer. The filtrate was then diluted with deionized water to provide the desired spore concentrations. *Aedes aegypti* used in these experiments were from the second and third generation of a colony derived from a field population in Dequincy, LA.

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Table 1. Effect of *per os* infection with *Edhazardia aedis* spores on *Aedes aegypti* larval mortality.

Diet (mg/larva)	Spore concentration (spores/ml)	Number of replicates	Percent larval mortality	
			Mean ± SD[1]	Range
0.8	0	5	75 ± 0.05[b]	68–82
0.8	1.5×10^3	10	60 ± 0.07[a]	48–72
0.8	1.5×10^5	10	81 ± 0.05[b]	70–88
3.2	0	5	2 ± 0.02[a]	0–4
3.2	1.5×10^3	10	16 ± 0.08[b]	4–28
3.2	1.5×10^5	10	2 ± 0.02[a]	0–6

[1] Within each diet treatment, average percent larval mortality with different letters are significantly different ($P \leq 0.05$, ANOVA).

The effect of oral infection on larval mortality and adult body size was examined by rearing *Ae. aegypti* in 2 concentrations of spores and with 2 diet regimes known to produce different adult body size distributions (Nasci 1990). Spore concentrations used were 1.5×10^3 spores/ml and 1.5×10^5 spores/ml. Larval diets consisted of 0.8 mg ground liver powder/larva and 3.2 mg ground liver powder/larva placed in the bowls at the beginning of the experiment. To ensure a constant particle size, liver powder was passed through a 100 mesh screen prior to suspension in water.

Larvae were reared in 11.5 cm diam × 5 cm deep glass bowls. Fifty 2nd instars (24 h post-hatch) were placed in each bowl with 200 ml of the spore suspension. The bowls were kept in an incubator at 27 ± 1°C, and a 16L:8D light/dark schedule. Each spore concentration and diet combination was replicated 10 times. Larvae subjected to the diet treatments but not exposed to spores served as controls, and were replicated 5 times for each diet level. Adults from the spore-treated bowls that did not become infected with the parasite also served as controls for the body size comparisons.

Percent larval mortality was calculated by counting and removing pupae from the bowls daily for 15 days. Pupae were placed in cages and held until adult emergence. The right wing of each adult male and female mosquito was measured to determine adult body size. Each adult was then smeared on a glass slide, fixed for 2 min in absolute methanol, stained for 10 min in 10% Giemsa, rinsed in deionized water and examined with a compound microscope for the presence of parasites.

The duration of infectivity of uninucleate spores removed from live, infected larvae and stored in water was examined. Two liters of a 1.5×10^5 spores/ml suspension in deionized water were prepared. The suspension was placed in a stoppered flask and held at room temperature (ca. 19°C). At intervals of 0, 12, 24, 36, 48, 60 and 72 h, the suspension was shaken and 200 2nd instar larvae were placed in 200 ml of the suspension with ample larval food. Larvae were reared to the 4th instar, and 20 larvae were smeared on glass slides, fixed and stained as described above. These smears were examined, and the percentage of larvae infected with the parasite at each storage interval was calculated.

Duration of spore infectivity during desiccation was studied by rearing transovarially infected larvae to the 4th instar. When numerous spores were present, a sample of the larvae was used to make a 1.5×10^5 spores/ml suspension. The rest of the larvae were placed on glassine paper, and dried at 22°C and 60% RH. A sample of 200 uninfected 2nd instar larvae was placed in 200 ml of the spore suspension and reared to the 4th instar. Twenty of the larvae were smeared, stained and observed for the presence of parasites to ensure that the spores were infective. Daily, for 7 days following drying, a suspension of 1.5×10^5 spores/ml was prepared from the dried spores and 200 uninfected 2nd instar larvae were placed in 200 ml of the suspension. These larvae were then reared to the 4th instar and 20 larvae were prepared for examination as above.

Larval mortality and mosquito wing lengths were compared with ANOVA. The larval mortality percentages were transformed with the arcsine transformation prior to statistical testing (Sokal and Rohlf 1981). Percentages of adults infected were compared using the Test for Equality of Percentages (Sokal and Rohlf 1981).

RESULTS

The effects of diet and oral infection with *E. aedis* uninucleate spores on *Ae. aegypti* larval mortality are shown in Table 1. Larval mortality in the 0.8 mg/larva diet was significantly greater than in the 3.2 mg/larva diet, both in the controls not exposed to spores, and compared within spore concentrations (ANOVA $P \leq 0.05$). Within the 0.8 mg/larva diet group, percent

Table 2. Percentage of adults infected with *Edhazardia aedis.*

Diet (mg/larva)	Spore concentration (spores/ml)	*n*	Number (%) infected
0.8	1.5×10^3	80	40 (50.0)*
0.8	1.5×10^5	43	13 (30.2)
3.2	1.5×10^3	200	70 (35.0)
3.2	1.5×10^5	335	198 (59.1)*

* Percentage infected significantly higher, within each diet treatment (Test for Equality of Percentages $P \leq 0.05$).

Table 3. Average wing length of *Edhazardia aedis* infected and uninfected *Aedes aegypti* adults.

Diet (mg/larva)	Spore concentration (spores/ml)	Mean wing length (mm) ±SD[1]			
		Female		Male	
		Infected (*n*)	Uninfected (*n*)	Infected (*n*)	Uninfected (*n*)
0.8	0		2.62 ± 0.15[a] (24)		2.00 ± 0.1[b] (38)
0.8	1.5×10^3	2.5 ± 0.07[a] (20)	2.54 ± 0.01[b]* (20)	2.16 ± 0.01[a] (20)	2.22 ± 0.05[a]* (20)
0.8	1.5×10^5	2.4 ± 0.07[b] (8)	2.54 ± 0.06[b]* (15)	1.90 ± 0.07[b] (5)	1.98 ± 0.01[c]* (15)
3.2	0		3.39 ± 0.16[a] (245)		2.58 ± 0.13[a] (222)
3.2	1.5×10^3	2.99 ± 0.06 (20)	3.18 ± 0.01[b]* (70)	2.41 ± 0.06[a] (50)	2.46 ± 0.01[b]* (60)
3.2	1.5×10^5	3.02 ± 0.11 (100)	3.07 ± 0.01[c]* (70)	2.38 ± 0.01[b] (98)	2.42 ± 0.01[c]* (67)

[1] Within sexes and diet treatments, among spore concentrations (columns), wing lengths followed by different letters are significantly different (ANOVA $P \leq 0.05$). Within sexes, diet treatments and spore concentrations (rows) wing lengths followed by an asterisk (*) are significantly greater (ANOVA $P \leq 0.05$).

mortality in the 1.5×10^3 spores/ml treatment was significantly lower than in the control or high spore concentration groups. Percent mortality did not differ between the control and high spore concentration groups within the low larval diet treatment.

Within the 3.2 mg/larva diet treatments, percent larval mortality was significantly higher in the 1.5×10^3 spores/ml group than in the control or 1.5×10^5 spores/ml group. Larval mortality was the same in the control and high spore concentration treatments within this diet level.

The percentage of adults that was infected differed between spore concentration treatments (Table 2). Within the 0.8 mg/larva diet, the percentage infected in the lower spore concentration was significantly greater than in the higher concentration. The opposite was found in the 3.2 mg/larva diet treatment in which the percentage of adults infected was significantly greater in the higher spore concentration.

The effects of diet and oral infection with spores on adult body size are shown in Table 3. Females and males reared on the 3.2 mg/larva diets had significantly longer wing lengths than those from the 0.8 mg/larva treatments. This was true of the controls, and of comparisons within spore treatment groups (ANOVA $P \leq 0.05$).

Within each diet and spore treatment, the average wing length of uninfected females and males (those not containing parasites when smeared as adults) was significantly longer than the wing length of infected individuals of the same sex (Table 3). Also within each diet treatment, the average wing length of the infected individuals from the lower spore concentration was significantly greater than from the higher spore concentration, except in the 3.2 mg/larva diet females where there was no difference.

Within the uninfected females of both diet levels, those from the control had significantly longer wing lengths than those that were exposed to spores. Also, the average wing length of uninfected females from spore treatments decreased with increasing spore concentration. The same pattern was seen in males from the 3.2 mg/larva diet but not in the lower diet level.

Spores held in water for periods of time after liberation from live, infected larvae quickly became non-infective to *Ae. aegypti* 2nd instars (Fig. 1). The freshly prepared spore suspension infected 65% of the mosquitoes, but infected none of the larvae by 48 h.

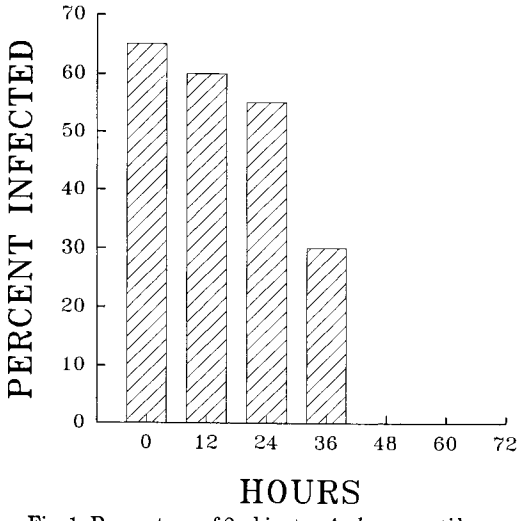

Fig. 1. Percentage of 2nd instar *Aedes aegypti* larvae infected after exposure to spores liberated from infected larvae and stored in water for intervals up to 72 h.

Drying larvae that contained spores rendered the spores noninfective to mosquito larvae when the spores were rehydrated and suspended in water. Spores from the fresh (undried) larvae infected 60% (12/20) of the larvae examined. After one day of drying, and at all subsequent intervals up to 7 days, none of the 20 larvae examined were infected with *E. aedis*.

DISCUSSION

Larval infection and mortality rates produced by the 10^3 to 10^5 spores/ml concentrations used in these experiments were considerably lower than previously reported for this parasite in *Ae. aegypti*. Hembree (1982) obtained 100% infection of 2nd instar *Ae. aegypti* with 10^3 spores/ml and 100% mortality with 10^5 spores/ml. The highest larval infection rate we obtained with 10^5 spores/ml was 65%. The highest larval mortality rate produced was 88% in nutritionally deprived larvae. Mortality did not exceed 6% in well fed larvae exposed to 1.5×10^5 spores/ml.

Differences in spore handling methods may have accounted for the reduced larval infection and mortality rates, though the methods described by Hembree (1982) do not appear to differ from those used in the present study. Better separation of spores from mosquito tissue debris may enhance infectivity. In addition, the presence of small suspended food particles in the bowls may have resulted in ingestion of fewer spores by the larvae. Hembree (1982) indicated that small amounts of food were added along with the spores, but the volume of food was not stated. A less likely explanation for the differences in infectivity and mortality are that the strain of *Ae. aegypti* used in this study may have been less susceptible to the parasite, or that continued laboratory colonization of the parasite may have altered its infectivity and pathogenicity.

Larval diet exerted more influence on larval mortality than did exposure to the parasite. Mortality rates were very high in the lower diet, regardless of the spore concentration. Within the lower diet level, larval mortality was significantly less in the low spore concentration treatment than in the high spore concentration, suggesting that excess mortality was caused by the increased parasite concentration. However, mortality in the high spore concentration group was the same as in the controls, indicating that the parasite did not produce any additional mortality. The reduced mortality in the low spore concentration group may have been caused by parasite-induced mortality in early instars reducing competition for food, thus increasing resources for the surviving larvae. A similar increase in survival rates resulting from "thinning" the population was described by Agudelo-Silva and Spielman (1984) as a probable result of inefficient larviciding, and was documented by Hare and Nasci (1986) and Washburn et al. (1991). The higher level of mortality in the control was probably due to competition for food; and in the high spore concentration group, higher mortality was probably due to the increased parasite load.

Within the 3.2 mg/ml larval diet level, larval mortality in the control and in the high spore concentration treatment were statistically the same, and were lower than mortality in the low spore concentration treatment. These results were unexpected since mortality usually increases linearly in response to spore concentration (Hembree 1982). However, some of the spores were infective, as indicated by the 35% infection rate in the adults from this treatment. Nutrition may play a significant role in the response of *Ae. aegypti* to this parasite, but these results are difficult to explain.

The percentage of infected adults within the 3.2 mg/larva diet was significantly greater in the high spore concentration than in the low spore concentration, indicating that the adult infection rate increased as spore concentration increased. However, the opposite was seen in the nutrient deprived treatment. This pattern could result if larval mortality due to the parasite was greater in the starved larvae. Therefore, at the low spore concentration, a greater proportion of the infected larvae survived to the adult stage, while larval mortality was higher and fewer in-

fected larvae survived at the high spore concentration.

Similar to the larval mortality pattern, adult body size was influenced more by larval diet level than by the parasite. Adults from the 3.2 mg/larva diets were larger than those from the 0.8 mg/larva diet. However, the parasite did cause a significant decrease in body size. In both diet treatments, mosquitoes that became infected and survived to the adult stage were significantly smaller than mosquitoes that were not infected. This observation is important because *Ae. aegypti* body size is related to several fitness qualities such as fecundity (Christophers 1960), blood-feeding success and survival in the field (Nasci 1986) and blood-feeding persistence (Nasci 1991). Therefore, the reduction in body size indicates that *per os* infection with the parasite reduces the overall fitness of the mosquito host.

The observation that control females were significantly larger than uninfected females from spore-treated bowls within each larval diet level suggests that exposure to the parasite without successful infection had an influence on the resulting adult. It is possible that some of the females in the spore-treated bowls became infected as larvae, recovered and emerged without evidence of the infection in the adult stage, and had a smaller body size as a result. No similar pattern is seen in the males.

Storage of uninucleate spores either in deionized water or in dried larvae quickly reduced the *per os* infectivity of *E. aedis* for *Ae. aegypti.* Though the conditions were very artificial, as natural *per os* infection probably involves larval feeding on infected cadavers in the aquatic habitat, this suggests that spores are infective for very short periods in the water, and that survival during drying is probably accomplished by transovarially infected eggs laid in the containers. Little is known about the stability of spores in field conditions. Since inoculating larval habitats with spores is one strategy for introducing *E. aedis* into the field, methods must be developed to prolong the viability of stored spores.

The results of this project indicate that interactions between *E. aedis* and *Ae. aegypti* are complex, and are influenced by larval diet. However, the reduction in adult fitness indicated by the decrease in the size of infected adults suggests that *E. aedis* has debilitative effects that may be utilized in a control program. Additional research to quantify the effect of *E. aedis* on *Ae. aegypti* and to develop parasite handling and storage methods is needed to determine the applicability of this parasite as a control mechanism.

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