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Epizootiological Studies of *Amblyospora albifasciati* (Microsporidiida: Amblyosporidae) in Natural Populations of *Aedes albifasciatus* (Diptera: Culicidae) and *Mesocyclops annulatus* (Copepoda: Cyclopidae) in a Transient Floodwater Habitat

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The epizootiology of the microsporidium *Amblyospora albifasciati* was studied in natural populations of its definitive host, a multivoltine, neotropical, floodwater mosquito, *Aedes albifasciatus*, and its intermediate copepod host, *Mesocyclops annulatus*, in an ephemeral floodwater habitat during a 12-month period. *A. albifasciati* was enzootic in mosquitoes. Vertically (transovarially) transmitted meiospore infections occurred regularly and were detected in five of eight larval broods but the prevalence of infection was always low, ranging from 0.5 to 6.9% with an overall average of 0.7%. Horizontal transmission of *A. albifasciati* infection from copepods to mosquitoes was nominal and limited. It was detected at levels of 6.4 to 20% in larval *Ae. albifasciatus* populations on two occasions, the month of August and late September through early October. The low levels of horizontal transmission of infection to mosquito larvae appeared to be the principal limiting factor that prevented the proliferation of *A. albifasciati* in *Ae. albifasciatus* populations. Copepod populations were abundant from May through September and weekly prevalence rates of *A. albifasciati* averaged over 50% (range = 5.8 to 100%). The moderately high infection rates in *M. annulatus* copepods were inconsistent with the low prevalence of meiospore infection in *Ae. albifasciatus* mosquito larvae. Results suggest that either meiospores of *A. albifasciati* produced in the mosquito host are highly infectious to copepods or they are long-lived and remain viable within the pool as long as some standing water is present. Observations further indicate that *A. albifasciati* has a significant detrimental impact on *M. annulatus* copepod populations but minimal impact on larval populations of *Ae. albifasciatus* at this site. © 2001 Academic Press

***Key Words: Amblyospora albifasciati;* Microsporidia; *Aedes albifasciatus;* mosquito; *Mesocyclops annulatus;* copepod; epizootiology.**

INTRODUCTION

Microsporidia of the genus *Amblyospora* are among the most common and widely distributed parasites that infect natural populations of mosquitoes. Over 90 species or isolates have been described from eight different genera of mosquitoes on five continents (Hazard and Chapman, 1977; Andreadis, 1994). Detailed life cycle studies have firmly established that most, if not all, species of *Amblyospora* undergo polymorphic development, are transmitted both vertically (transovarial) and horizontally (oral), and utilize copepods as intermediate hosts (Andreadis, 1985b, 1988, 1990; Sweeney *et al.*, 1985, 1988; Becnel, 1992; White *et al.*, 1994; Micieli *et al.*, 1998, 2000). In addition, these microsporidia appear to be highly specific for the definitive mosquito host but less so for the intermediate copepod host (Andreadis, 1989; Sweeney *et al.*, 1990; Becnel, 1992; Becnel and Andreadis, 1998).

Despite their prevalence and widespread distribution in mosquitoes, few studies have examined the epizootiology of *Amblyospora* infections over time and space in wild mosquito populations (Welch, 1960; Saba *et al.*, 1984; Andreadis, 1999). Moreover, because of the complexity of the life cycle and difficulty in identifying the intermediate host, only one study (Andreadis, 1990) has examined the concurrent epizootiological dynamics of infection in both mosquito and copepod populations.

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Amblyospora albifasciati is a recently described parasite from a neotropical floodwater mosquito from Argentina, *Aedes albifasciatus* (Garcia and Becnel, 1994). It has a typical "*Amblyospora*" life cycle wherein adult female *Ae. albifasciatus* transovarially transmit infections to filial generations of larvae via ovarian infection by binucleate spores. Parasite development in transovarially infected larvae occurs within fat body tissue and culminates in the production of tens of thousands of haploid meiospores. Meiospores are liberated into the aquatic environment with the death of infected larvae and these are perorally infectious to female stages (copepodite and adult) of the cyclopoid copepod, *Mesocyclops annulatus*. A third spore type (uninucleate) is produced in ovarian tissue of the copepod and these are similarly released into the water with the death of the copepod. Uninucleate spores are responsible for horizontal transmission of *A. albifasciati* to larval *Ae. albifasciatus* via oral ingestion. Mosquito larvae thus infected develop benign infections which eventually lead to the production of binucleate spores in adult female hosts and transovarial transmission to the next generation of mosquito larvae (Micieli *et al.*, 2000).

The present study was undertaken to gain a better understanding of the natural epizootiological dynamics of this complex host-parasite system in an ephemeral floodwater habitat. Our specific objectives were to quantify the seasonal prevalence of *A. albifasciati* infection in populations of its definitive mosquito host, *A. albifasciatus*, and its intermediate copepod host, *M. annulatus*, over a 12-month period, assess the impact of the microsporidium on host populations, and elucidate the transmission and survival strategies employed by *A. albifasciati*.

MATERIALS AND METHODS

Biology of Ae. albifasciatus

Ae. albifasciatus is a multivoltine, floodwater mosquito that is widely distributed throughout Argentina (Prosen *et al.*, 1960; Forattini, 1965). Larvae develop in ephemeral pools in low-lying wetlands. *Ae. albifasciatus* survives dry periods (up to 6 months) in a dormant egg stage. Egg hatch typically occurs whenever pools are flooded and as many as eight broods a year may be produced during the rainy season (May through February). Larval development is approximately 9 days at 23°C. Adult females are long-lived and have been shown to complete up to five gonotrophic cycles, suggesting an estimated survival of 35–50 days (Luduena Almedida, 1995).

Collection Methods and Assay Procedures

A characteristic, grassy pool habitat, approximately 100 × 100 m was selected for this study. The area was located 10 km west (34° 57′ 89″S, 58° 03′85″W) of La Plata City, Buenos Aires Province, Argentina. This site became flooded exclusively with heavy local rainfall. Stakes were placed around the perimeter of the pool at time of maximum flooding. Water levels were then quantified weekly by calculating relative surface area as a percentage of the maximum total area achieved with full (100%) inundation.

The site was sampled weekly from May 1996 through May 1997 and sampling for immature *Ae. albifasciatus* and *M. annulatus* copepods was conducted whenever water was present. Specimens were collected with a standard 300-ml dipper and 100 sample dips were made on each occasion. Samples were immediately transported to the laboratory for examination.

All *Ae. albifasciatus* larvae were examined for horizontally and vertically acquired infections with *A. albifasciati* and the overall prevalence of each type of infection was recorded. Mosquito larvae were initially screened against a black background for patent fat body infections (vertically transmitted) under a stereomicroscope. Confirmation of infection was based on the presence of meiospores as observed in wet mount dissections of infected tissues under phase-contrast microscopy (400×). Larvae not exhibiting overt symptoms of infection were collectively maintained at 26 ± 1°C until pupation. Giemsa-stained (10%, pH 7.41) smears were made of individual pupae and these were microscopically examined (1000×) for evidence of horizontal transmission (i.e., uninucleated stages).

Copepods were identified using keys of Reid (1985) and Ringuelet (1958) and the presence and relative abundance of adult female and copopodite stages of *M. annulatus* were recorded. The prevalence of *A. albifasciati* within the copepod sample was similarly determined from microscopic examination of Giemsa-stained smears of a variable number of copepodite and adult female stages for vegetative stages and/or haploid spores that were readily recognizable.

RESULTS

The pool was inundated with floodwater on 11 separate occasions during the 12-month study (Fig. 1A). The first brood of *Ae. albifasciatus* larvae was recorded in early May (Fig. 1B). A week later most larvae had developed to the fourth stadium and 2% (n = 100) were infected with meiospores (vertical transmission) of *A. albifasciati* (Fig. 1C). No mosquitoes were found after the second week. Adult *M. annulatus* were similarly recovered in early May but unlike mosquitoes, they continued to be abundant throughout the month (Fig. 1B). Weekly prevalence rates of *A. albifasciati* in adult female *M. annulatus* declined from 36.4 to 5.5% with an overall average of 20.8% (n = 101) (Fig. 1C). Two-thirds of the infected

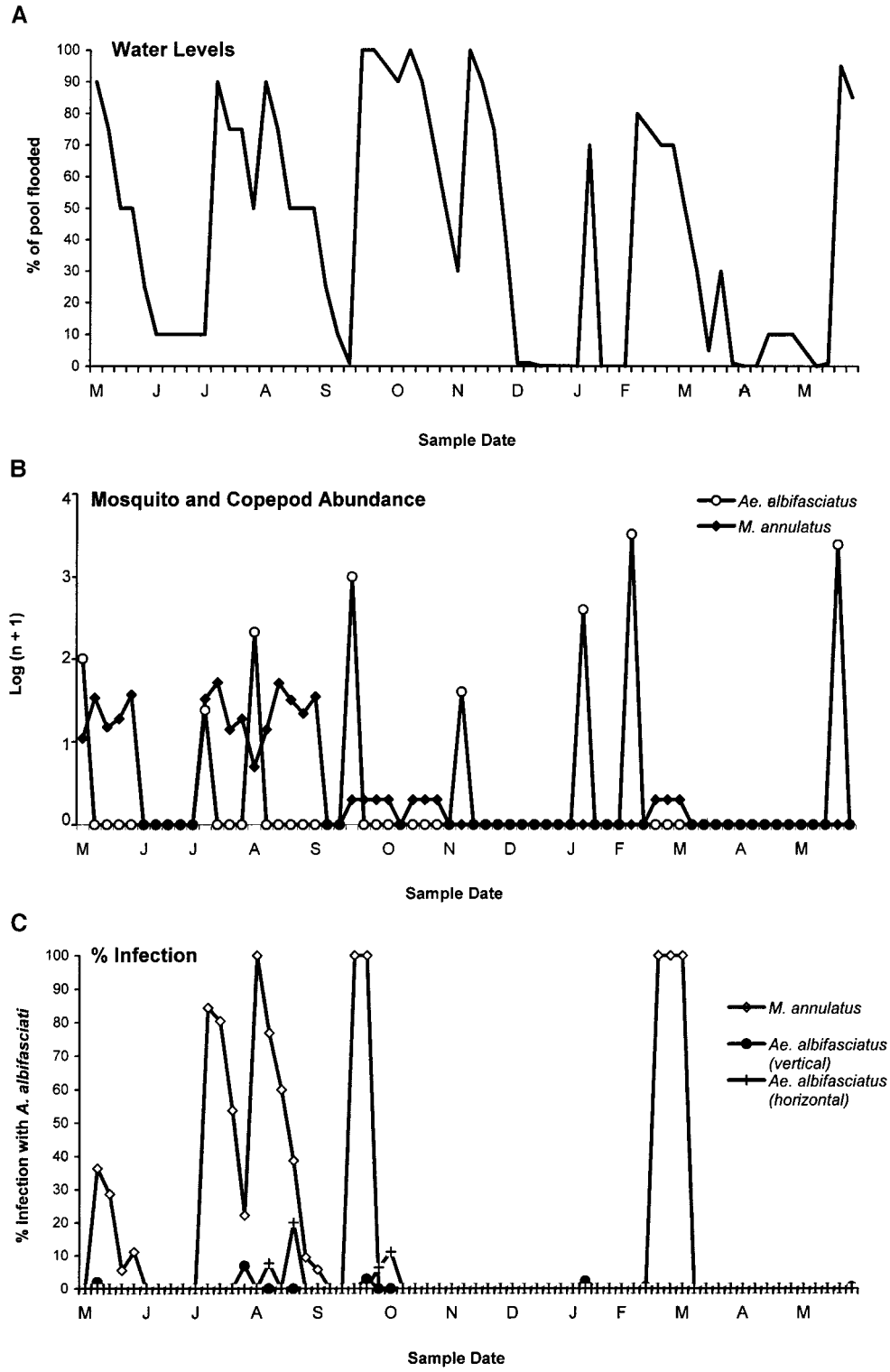

FIG. 1. Seasonal abundance of *Aedes albifasciatus* mosquitoes and *Mesocyclops annulatus* copepods and prevalence of *Amblyospora albifasciati* infection in a transient floodwater habitat in La Plata, Argentina. (A) Water levels within the pool (% flooded). (B) Abundance of first-instar *Ae. albifasciatus* (○) and adult female *M. annulatus* (◆). (C) Prevalence of *A. albifasciati* infection in *M. annulatus* (◇) and larval *Ae. albifasciatus* (horizontally transmitted (+) and vertically transmitted (●)).

copepods were found to harbor only vegetative stages. Water levels in the pool steadily receded throughout May and by June only 10% of the pool remained flooded.

The site was reflooded on July 8. A very high prevalence of *A. albifasciati* infection (84%, all spores, n = 32) was immediately detected in adult *M. annulatus* copepods (Fig. 1C). These prevalence rates in copepods remained high for one additional week (80.4%, n = 52) and then gradually declined to 22% as the copepod population density waned and water levels receded. A second generation of *Ae. albifasciatus* larvae was observed at the same time but no infections with *A. albifasciati* were detected until the end of July when 6.9% of the fourth-instar larvae (n = 29) were found infected with meiospores indicative of vertical transmission.

In early August, new rains increased the flooded area of the pool to 90%. A third brood of first-instar *Ae. albifasciatus* larvae was recorded along with a small number of copepods (n = 4), all of which were infected with *A. albifasciati*. The first horizontally transmitted infections of *A. albifasciati* in *Ae. albifasciatus* were detected during the first 3 weeks of August. Prevalences ranged from 7% (n = 13) to 20% (n = 30). Copepod populations increased following the flooding. The prevalence of *A. albifasciati* remained relatively high in copepods throughout August, but steadily declined as water levels receded. The overall infection rate in copepods was 40.6% (n = 192) during this flooded period. The pool was completely dry by early September.

The area was reflooded on September 17. First-instar *Ae. albifasciatus* larvae were observed the following day. One week later, 2.9% (n = 103) of the larval mosquito population was infected with meiospores. Horizontally transmitted infections were also detected in *Ae. albifasciatus* larvae for the second time. Prevalences ranged from 6.4% (n = 62) in late September to 11.1% (n = 9) in early October. Very few copepods were observed, but all examined from mid to late September were infected.

A major flooding (100% inundation) occurred in November and a fifth brood of *Ae. albifasciatus* larvae was recorded, but no copepods were observed. No *A. albifasciati* infections were detected in any *Ae. albifasciatus* larvae.

The pool was completely dry from December until the middle of January when rains brought the flooded area to 70%. A sixth brood of first-instar *Ae. albifasciatus* larvae was recorded and 1 week later meiospores were observed in 2.2% (n = 400) of fourth-instar larvae. No *M. annulatus* copepods were recovered during this flooding.

The pool was reflooded in February (90%) and 1 week later 0.5% (n = 3278) of *Ae. albifasciatus* larvae were found infected with meiospores. Only one *M. annulatus* copepod infected with vegetative stages was collected at this time. Water levels steadily declined through March and April during which no mosquitoes or copepods were recovered.

The site was flooded again on May 22 and a final collection was made. Large numbers of *Ae. albifasciatus* larvae were collected (n = 2446) and 0.5% were infected with meiospores. No copepods were found.

DISCUSSION

The ephemeral nature of this flood water habitat for *Ae. albifasciatus* was markedly evident in the present investigation. The pool was subject to periodic flooding due to local rainfall and was inundated on 11 separate occasions during the 12-month period. This resulted in eight distinct broods of *Ae. albifasciatus*, as indicated by the presence of first-instar larvae, but only when water levels exceeded 70% of the total area of the pool. This latter finding reaffirmed a previous unpublished observation (M. Micieli) that adult female *Ae. albifasciatus* mainly oviposit along a 10-m strip surrounding the outer perimeter of the pool.

A. albifasciati was clearly enzootic. Transovarially transmitted infections were detected in five of the eight larval broods of *Ae. albifasciatus*. These were regularly noted in fourth-instar larvae 5 to 10 days after flooding and resulted in death of infected individuals prior to pupation as noted in a previous laboratory study (Garcia and Becnel, 1994). The prevalence of infection, however, was repeatedly low ranging from 0.5 to 6.9% with an overall average of 0.7% (n = 4900) for the entire sampling period. These findings are consistent with the enzootic nature of most *Amblyospora* spp. and the typically low levels of infection observed in larval mosquito populations (Welch, 1960; Kellen and Wills, 1962; Chapman *et al.*, 1967, 1969; Anderson, 1968; Andreadis, 1994, 1999; Garcia and Becnel, 1994).

Despite the repeated occurrence of transovarially infected larvae, no seasonal or generational epizootics were observed in the mosquito host as has been noted with *Amblyospora connecticus* in *Aedes cantator*, a multivoltine coastal salt marsh mosquito that develops in a similarly unstable, transient habitat that is subject to periodic flooding and drying (Andreadis, 1990). In this case, transovarially infected eggs of *Ae. cantator* hatch synchronously in the fall in concert with the reemergence of aestivating copepods. Although the physiological mechanisms responsible for timing of this hatch are unknown, this phenomenon facilitates horizontal transmission of infection to copepods by ensuring that a maximum inoculum of meiospores is produced when susceptible hosts are present. There was no evidence to indicate that a similar phenomenon operates in the epizootiological dynamics of *A. albifasciati*. We further con-

clude that while transovarial transmission of infection occurred regularly, *A. albifasciati* had minimal impact on larval populations of *Ae. albifasciatus* at this site. Complementary studies in other habitats could be done to more fully assess the epizootic potential of this microsporidium. However, repeated multiyear investigations with other mosquito parasitic microsporidia (Saba *et al.*, 1984; Andreadis, 1985a, 1990, 1999) have generally revealed similar patterns of infection and transmission year after year.

Horizontal transmission of *A. albifasciati* infection from spores formed in copepods to mosquitoes was nominal and limited. It was detected in larval *Ae. albifasciatus* populations on two occasions only, early to late August and in late September through early October. The apparently low level of horizontal transmission of infection to mosquito larvae appeared to be the principal limiting factor that prevented the proliferation of *A. albifasciati* in *Ae. albifasciatus* populations, but it was seemingly not due to a paucity of infected copepods. In both instances, horizontal transmission to mosquito larvae was preceded by moderately abundant populations of *M. annulatus* copepods within the pool and the presence of relatively high infection rates therein (80 to 100%). However, despite the apparent profusion of infectious spores produced in these copepods, the subsequent infection rates in mosquitoes never exceeded 20%, thus suggesting low infectivity to larvae. This conclusion is consistent with the low levels of transmission (9.7%) achieved when mosquito larvae are exposed to spores from *M. annulatus* in controlled laboratory bioassays (Micielli *et al.*, 2000). On the other hand, we cannot rule out other environmental or host (behavioral or physiological) factors that could also negatively affect transmission.

The low levels of horizontal transmission observed with *A. albifasciati* in the present study contrast sharply with the comparatively high prevalences of horizontally transmitted infections seen with *A. connecticus*, which typically average 40% and have been reported to be as high as 60% in first-generation *Ae. cantator* populations in the spring when infected (40 to 75%) copepods are abundant (Andreadis, 1994). However, the horizontal transmission rates observed in *Ae. albifasciatus* were still notably higher than those reported annually for *Amblyospora stimuli* (0.1 to 8.7%), which infects *Aedes stimulans*, a univoltine mosquito that inhabits a temporary vernal pool that is flooded once yearly. In that habitat scarce copepod populations are generally thought to be the major factor that limits the proliferation of the microsporidium in mosquito populations (Andreadis, 1999).

The high prevalences of infection in *M. annulatus* copepods observed in this study were inconsistent with the exceedingly low levels of meiospore infection noted in *Ae. albifasciatus* mosquito larvae. Although a small number of infected mosquito larvae were generally found whenever copepods were present, it is difficult to envision how the seemingly low meiospore inoculum could result in such high prevalences of infection in *M. annulatus*. This suggests that either meiospores of *A. albifasciati* are highly infectious to copepods or they are long-lived and remain viable within the pool as long as some standing water is present. Although water levels fluctuated greatly in this habitat, some standing water was evident most of the year and on only one occasion did the pool completely dry out during the 7-month period from May through November. Andreadis (1991) has shown that meiospore infectivity for copepods with *A. connecticus* can be maintained for up to 5 months when meiospores are held in water at 4°C. In preliminary laboratory bioassays with *A. albifasciati*, the senior author (M.V.M.) has observed meiospore infectivity for copepods for at least 15 days at 20°C, but further studies are needed.

It is also possible that *A. albifasciati* may have killed copepods slowly and retarded development resulting in a biased sample of infected females. This hypothesis is supported by the detection of vegetative stages of *A. albifasciati* in copepod populations only during May, while at other times of the year (July through November) they were always found infected with mature spores. The presence of vegetative stages most likely indicates a newly acquired infection while the presence of mature spores indicates a longer term infection. In the laboratory, *M. annulatus* copepods infected with *A. albifasciati* may remain alive up to 35 days postinfection (M. Micielli, unpublished) and meiospore infections in mosquitoes are known to prolong larval life beyond that of the uninfected portion of the population (Andreadis, 1993). Comparative studies on copepod longevity should be similarly conducted to assess this possibility.

One other possible explanation for the high infection levels in *M. annulatus* is that copepods are preferentially attracted to feed on meiospore-infected cadavers of *Ae. albifasciatus* that come to rest on the bottom of the pool. This would presume directed browsing behavior in the bottom sediment by *M. annulatus* and the possible emission of a chemical stimulus from infected cadavers. Recent studies have shown that copepods can detect food particles through the use of chemo- and mechanoreceptors (Dussart and Defaye, 1995) but we have no knowledge of host-directed chemical attractants of microsporidian origin.

While it was evident that transstadial transmission of infection occurs from copepodid to adult, transovarial transmission of infection in copepods can be ruled out as contributing to the high infection rates, since infections occur within the ovary and prohibit egg development (Micieli *et al.*, 2000).

Our data further suggest that while *A. albifasciati* appeared to have little effect on larval populations of *Ae. albifasciatus*, it may have had a significant detrimental impact on *M. annulatus* copepod populations at this site. Few copepods were found in the pool from mid-September through early May. This followed a 4-month period during which exceptionally high infection rates were noted in copepod populations. During the same period (September–May), we monitored over 50 other pools in the same general area that served as breeding sites for larval populations of *Ae. albifasciatus*. No *A. albifasciati* infections were found in any *M. annulatus* copepods inhabiting these pools while they remained abundant throughout the year. Although further experimental studies are needed, these direct observations suggest that *A. albifasciati* may have been a significant factor contributing to the decline in copepod populations in this pool.

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