PERSISTENCE OF THE MOSQUITO FUNGAL PATHOGEN *LAGENIDIUM GIGANTEUM* (OOMYCETES; LAGENIDIALES) AFTER INTRODUCTION INTO NATURAL HABITATS[1]

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ABSTRACT. Two isolates (North Carolina and Louisiana) of *Lagenidium giganteum* were introduced into natural freshwater mosquito breeding sites in North Carolina in June 1982 and their establishment and persistence monitored through the remaining mosquito breeding season. The North Carolina isolate on agar media was introduced into a flooded woodland that had populations of *Anopheles punctipennis, Culex restuans, Cx. territans, Aedes vexans, Psorophora ferox* and *Ps. columbiae*. A liquid culture of the Louisiana isolate was added to a flooded depression that was a source of *An. punctipennis, Cx. restuans, Cx. territans* and *Ae. vexans*. The fungus became established at both sites, remaining at enzootic levels when larvae were scarce and recrudescing when larval populations increased after flooding. Infections during the observation period ranged from 0 to 100% among larvae collected from the sites and 20 to 100% among laboratory-reared larvae *(Cx. quinquefasciatus)* exposed in the sites for 24–48 hr. Mosquito larvae added to water samples drawn from the sites and held in the laboratory became infected with the fungus even when natural larval populations were very low or absent. Results indicate that *L. giganteum* recycled for the entire season despite periodic scarcity of hosts and short-term drought. When the water temperature dropped to below 18°C, infection of mosquito larvae by the fungus ceased.

INTRODUCTION

Current information about *Lagenidium giganteum* Couch indicates that the fungus has potential as a self-sustaining biocontrol agent of mosquitoes once it has been introduced into a suitable habitat (Christensen et al. 1977, Washino 1982). However, little published data exist on the persistence and infectivity for mosquito larvae of the fungus throughout a mosquito breeding season in natural habitats. Glenn and Chapman (1978) reported some observations of a natural epizootic in Louisiana. Artificial introductions in California (McCray et al. 1973, Christensen et al. 1977, Washino and Fukushima 1978), have been concerned primarily with short-term effects or the overwintering ability of *L. giganteum* in irrigated rice field and pasture habitats.

Our investigation concerns the introduction of *L. giganteum* into natural, semi-permanent habitats and observations on the persistence of the fungus and infection of mosquito larvae during periods of fluctuating mosquito populations and water levels. Results from trials with 2 isolates of the fungus are reported.

MATERIALS AND METHODS

MCALPINE GREENWAY PARK SITE (CHARLOTTE, NC)

Description. The site consisted of ca. 800 m^2 of standing water within a mixed deciduous forest. Water depth varied from 5 to 60 cm when the entire site was flooded. This area was a temporary mosquito breeding habitat in which the standing water fluctuated greatly in depth and coverage, yet persisted in at least several isolated pools for most of the summer. Mosquito larvae present included low numbers of *Anopheles punctipennis* (Say), *Culex territans* Walker, *Cx. restuans* Theobald with large broods of *Aedes vexans* (Meigen), *Psorophora ferox* (Von Humboldt) and *Ps. columbiae* (Dyar and Knab) after sufficient rainfall and flooding. At the time the fungus was introduced, water from this site had a Chemical Oxygen Demand (COD) of 60.5 mg/liter, Total Kjeldhal Nitrogen (TKN) of 1.82 mg/liter, Ammonia Nitrogen (NH_3.N) of 0.74 mg/liter, Total Phosphorous (TP) of 1.17 mg/liter and pH = 6.8, measured by standard techniques (American Public Health Assoc. 1976). These levels did not change appreciably during the subsequent season (Table 1). The water was non-saline (<0.2 ppt NaCl). Water temperature, measured at 3–7 day intervals with a maximum-minimum thermometer, fluctuated between 17° and 28°C during the field test. At the start of the trial the mean mosquito larval density was 3.6 per dip (140 dips). The pretreatment larval mosquito survey did not reveal any mosquito pathogens except a very low incidence of a microsporidium (*Amblyospora* sp.), 3 of 145 *Cx. restuans* examined.

Experimental Methods. The North Carolina (NC) isolate of *L. giganteum* was added to the site on June 8, 1982. The fungus was cultured in Z Medium, then plated onto hemp seed agar in standard 10 cm diam plastic petri dishes (Jaronski et al. 1983). Five-day old agar cultures of the fungus were introduced into 1 liter

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Table 1. Results of tests of water samples from the field sites.

Sample date	Water quality parameter				
	TKN (mg/l)	COD (mg/l)	NH_3.N (mg/l)	TP (mg/l)	pH
	McAlpine Greenway Park site				
June 8	1.82	60.5	0.74	1.17	6.8
June 26	1.60	55.0	0.68	1.00	6.7
July 25	1.79	72.3	0.68	1.20	7.0
	New Hope River site				
June 23	1.66	102.0	0.40	0.53	7.8
July 1	1.91	105.0	0.51	0.56	7.7
July 18	1.50	92.0	0.46	0.40	7.5

plastic enclosures (32 cm diam × 20 cm height with open bottom and top) set into one portion of the field site. The fungus from the petri dishes was minced and suspended in pond water and then added to each enclosure with dose rates equivalent to 3 petri dishes/m^2 for 6 enclosures and 6 petri dishes/m^2 for 5 enclosures. Along with the fungus introduction, 50 to 100 mosquito larvae (a mixture of *Cx. restuans, Cx. territans* and *An. punctipennis*) collected from the immediate area and 50 second- and third-instar *Cx. quinquefasciatus* Say from a laboratory colony were added to each enclosure. A floating screened cage containing 29 second-instar laboratory-reared *Cx. quinquefasciatus* larvae (sentinels) was placed into each enclosure.

A laboratory bioassay was conducted with cultures from the same batch of *L. giganteum*. In these bioassays, ½ of an agar dish culture was minced and added to a 39 × 24 cm enameled pan containing 3 liters of deionized water and the other half added to a pan with water from the site. The assays were replicated 3 times with 100 second-instar *Cx. quinquefasciatus* larvae from a laboratory colony added to each pan. Untreated controls consisted of 100 larvae in 3 liters deionized water or water from the sites (2 replicates each).

Our intent was to establish the fungus within the enclosures, then remove them and observe whether or not *Lagenidium* would spread throughout the entire site. However, on the second day after adding the fungus, rains flooded the area and swept away all enclosures and sentinel cages. Flood water peaked about 1.5 m above the original water level, then subsided within 12 hr. The monitoring scheme was altered to weekly collection of larvae and the exposure of sentinel larvae within a floating screen cage at each of 5 stations when standing water was present during the observation period. The 5 stations were established about 10 m apart at points along the long axis of the site. At each station 10 dips were made with a 450 ml dipper within a 4 m^2 area and the larvae collected were examined for infection 1 day later. A floating cage containing 20 third-instar *Cx. quinquefasciatus* was placed at each station for 48 hr. The larvae were examined for infection a day after removal from the site. Monitoring was continued until late August when the entire site dried out for the remainder of the mosquito breeding season.

At intervals during the trial, 2-liter samples of water from the site were collected and transported to the lab where 20 third-instar *Cx. quinquefasciatus* larvae were added and examined for infection 3 days later.

NEW HOPE RIVER SITE (DURHAM, NC)

Description. The habitat was a 2.6 m^2 irregularly shaped, grassy depression which was a semi-permanent breeding habitat for *Cx. territans, Cx. restuans* and *An. punctipennis* and for *Ae. vexans* after flooding by rains. The depression consisted of several centimeters of humus covering the pavement of an abandoned road and, when full, had a water depth of 5 to 10 cm. The water was relatively unpolluted, with COD = 102 mg/liter, TKN = 1.66 mg/liter, NH_3.N = 0.4 mg/liter, TP = 0.53 mg/liter and pH = 7.8 at the start of the field trial. These levels of water quality did not change substantially during the season (Table 1). The water was non-saline. Water temperature varied between 18° and 28°C during the months of June, July and August, but fell to a daily range of 13° to 18°C by October. At the start of the trial the mosquito larval density was 16 per dip (mean of 10 dips). The larvae were *Cx. territans* and *Cx. restuans;* 70% of the larvae were collected as first- and second-instars and the remaining 30% as third- and fourth-instars. The site was also surveyed for mosquito pathogens during the week preceding the introduction of the fungus and no pathogens were found.

Experimental Methods. The Louisiana (LA) isolate of *L. giganteum* grown from liquid culture was added to the site on June 23, 1982. The fungus had been grown 5 days in peptone-yeast-glucose broth, then subcultured in Z Medium (Domnas et al. 1982) for 5 days. Mycelium from 75 ml of culture (1 flask) was washed once in deionized water, homogenized very briefly to break up mycelial masses, then suspended in 100 ml deionized water. The fungal suspension was transported to the site, diluted with 2 liters of water from the site and sprayed over the entire depression from a hand sprayer (15 p.s.i.). The application rate was 30 ml of original culture per m^2 of habitat.

A second flask of the fungus was processed as described above and used in a laboratory bioas-

say with *Cx. quinquefasciatus* larvae. A dose of 1/4 (19 ml) and 1/16 (5 ml) of the culture were dispersed in 45 × 35 cm pans containing 8 liters of deionized water and 100 second-instar larvae with 2 replicates per dose. These were equivalent to the field rate of application and 1/4 the field rate, respectively. After 3 days the larvae were examined for presence of the fungus.

Prevalence of *Lagenidium* after introduction was monitored by collecting larvae and the use of sentinel larvae. Ten dip samples with a standard 450 ml dipper were made on the third day after introduction of *Lagenidium* and once a week thereafter. All larvae from those collections were held in the laboratory for 1 day and then examined microscopically for the presence of the fungus. Two floating, screened sentinel cages were placed in the pool immediately after the fungus was introduced. Each cage contained 20 second-instar *Cx. quinquefasciatus* and was kept in the water for 24 hr. The larvae were then removed to the lab, and held 36 to 48 hr before being examined microscopically for the presence of the fungus. Sentinel larvae were exposed in the pool once a week thereafter, while there was water in the site, in the manner just described. In addition, 1 liter water samples were drawn from the site weekly, 25 second-instar *Cx. quinquefasciatus* were added immediately and the samples were transported to the lab where the larvae were examined for infection after 2 to 3 days.

RESULTS

MCALPINE GREENWAY PARK SITE. No data were obtained for the initial infection rates in the enclosures because of the flood on day 2 of the experiment. Subsequent collections and sentinel larvae, however, demonstrated that *L. giganteum* had become established throughout the test site (Fig. 1). On day 17, 64% of collected larvae were infected when larval numbers in the site averaged 5.2 larvae/dip. This trend (relatively low numbers of larvae with a substantial percentage infected) continued for the next 19 days, when rainfall flooded the site and a brood of *Ae. vexans* and *Psorophora* spp. hatched. Transmission of *L. giganteum* rapidly increased and the mosquito brood rapidly reached almost 100% infection among collected larvae (see Fig. 1, day 38). Sentinel larvae placed in the site during this period, day 35 to day 45, revealed an infection level of 70% to 90% over 48 hr. Between day 46 and 55 the site was reduced to a few small pools of water. Larvae disappeared from the site, yet 20% of the sentinel larvae placed in the remaining pools became infected. On day 64, rain reflooded the site and caused another brood of *Ae. vexans* and *Psorophora* to hatch. Transmission of *L. giganteum* to sentinel larvae and to naturally present larvae increased but only to an endemic level (20% to 30%). By this time, water temperatures that had previously ranged from 20° to 28°C had decreased to

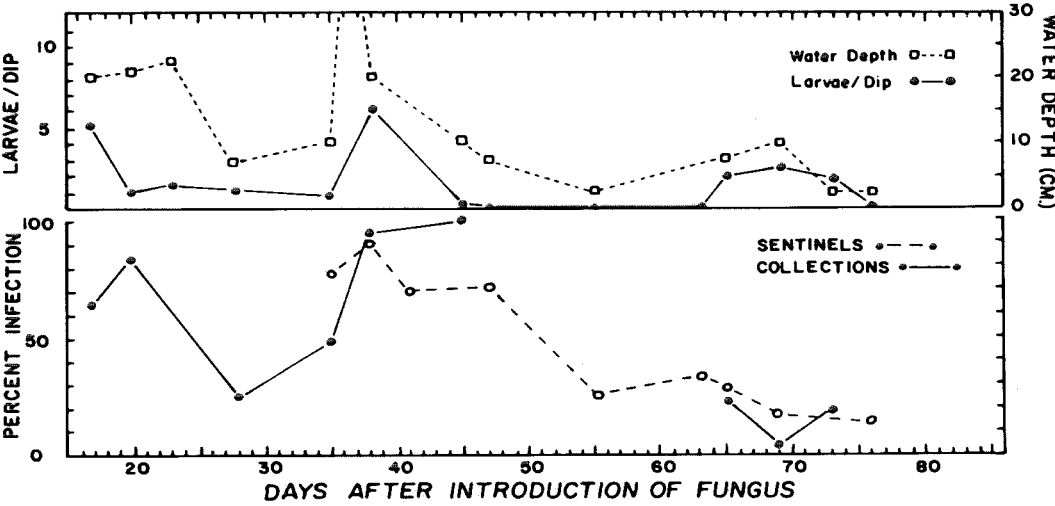

Fig. 1. Results of a field introduction of *Lagenidium giganteum* (NC isolate) into an 800 m² flooded woodland at McAlpine Greenway Park, Charlotte, NC. Upper graphs: Water depths and larval numbers in the site during the trial. Lower graphs: Infection rates among larvae collected from the site and sentinel larvae exposed for 48 hr in the site.

18°–22°C, the threshold for transmission of *L. giganteum* (Jaronski and Axtell 1983).

Water samples drawn from the site on different days possessed variable amounts of infective material (Table 2). The samples from days 49, 52, 61 and 78 were notable because mosquito larvae were rare in the site when the water samples were collected.

Table 2. Rates of infection by *Lagenidium giganteum* (NC isolate) of *Culex quinquefasciatus* larvae exposed to water samples from the McAlpine Greenway site at intervals after fungus introduction, June 8, 1982.

No. days after introduction of fungus	% infection*
49	58
52	90
61	95, 30
66	14, 0
78	75, 25

* Twenty-five second-instar larvae placed in 2 liter water sample. Where two percentages are given, each value represents a separate sample.

In the bioassay of the fungus inoculum used in this trial there was 93% infection of larvae in deionized water, 92% in site water and no infection in the corresponding controls without fungus.

NEW HOPE RIVER SITE. Three days after *L. giganteum* (LA isolate) was introduced into the site, 17% of collected larvae and 55% of sentinel larvae were infected (Fig. 2). Laboratory bioassay of the inoculum gave 82% infection at the field rate and 47% at ¼ the field rate with no infection among controls without the fungus. These infection rates were typical of laboratory bioassays. In subsequent days, prevalence and incidence levels remained high despite very low numbers of naturally present larvae. By day 28 the site had dried and larvae were absent. The prevalence and incidence levels also dropped. On day 37 rains flooded the site and caused a brood of *Ae. vexans* to hatch; 70% of this brood was infected by the fungus. During the next 11 days infection levels among sentinel larvae remained high even though larvae were rare or absent from the site. By day 57 of the trial the site dried out and remained dry for the next 50 days. In October the site was again flooded but no fungus was observed in either sentinel larvae or the larvae naturally present. Water temperature in the site had fallen to 16°C, which was below the threshold necessary for transmission of *Lagenidium*.

Culex quinquefasciatus larvae added to water samples from the site on 6 different days during the trial became infected after exposed for 48 hr (Table 3). The infection rate was 36% at 56 days after introduction of the fungus. The exceptionally high infection levels for larvae exposed to these samples indicated that large numbers of infective zoospores were present,

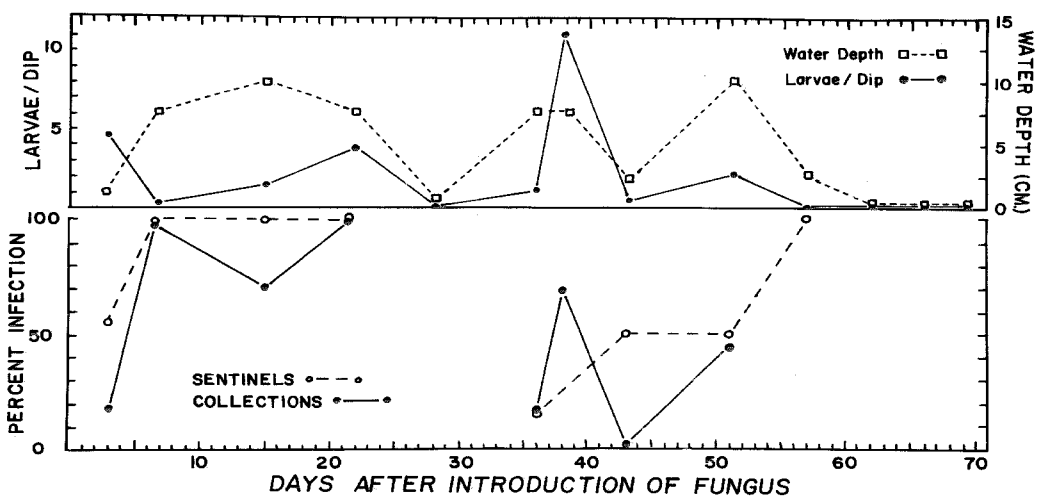

Fig. 2. Results of a field introduction of *Lagenidium giganteum* (LA isolate) into a 2.6 m² flooded depression in the New Hope River drainage area, Durham Co., NC. Upper graphs: Water depths and larval numbers during the trial. Lower graphs: Infection rates among sentinel larvae and larvae collected from the site.

Table 3. Rates of infection by *Lagenidium giganteum* (LA isolate) among *Culex quinquefasciatus* larvae exposed to water samples from the New Hope River site at intervals after fungus introduction, June 23, 1982.

No. days after introduction of fungus	% infection*
15	100
36	84
38	100
42	44
51	56
56	36

* Twenty-five second-instar larvae placed in 1 liter water sample.

even though natural mosquito populations were very small as on days 36, 42 and 56. Infection levels among sentinel larvae exposed on those days (Fig. 2) correlate with data from the water samples.

DISCUSSION

Given suitable temperature and water quality, both isolates of *L. giganteum* applied by different methods (solid agar and liquid culture) became established and persisted despite the scarcity or even absence of host larvae and survived at least short periods of drought. At both sites in this study *L. giganteum* infected a substantial proportion of the mosquito populations, even when larval numbers were comparatively low (<1 larva/dip). High infection levels of sentinel larvae exposed at the sites, as well as infectivity of water samples drawn from the sites, demonstrated that the fungus was releasing infective zoospores even when larvae were rare, and moreover, maintaining itself at enzootic levels. When mosquito broods appeared after the sites had been flooded. *L. giganteum* attained epizootic levels and infected 70–100% of the newly hatched *Ae. vexans, Ps. ferox* and *Ps. columbiae* within 48 hr after brood hatch. We were not able to document the extent of control, because mosquito production from adjacent control areas was very erratic. Since mortality from infection by *L. giganteum* is 100%, however, the prevalence of the fungus in the mosquito populations was a good indicator of control effectiveness. Larvae from McAlpine Greenway Park site exhibited 0–100% infection. These data may be underestimates because infected larvae were rapidly consumed by saprophytic organisms after death and by predators while still alive. Data from sentinel larvae probably were more reflective of actual transmission rates.

As we have observed in previous field trials (Jaronski and Axtell 1982), fungal transmission ceased with the onset of cooler water temperatures in the fall. Laboratory experiments (Jaronski and Axtell 1983) revealed that temperatures below 18° to 20°C were inimical to continued transmission of *L. giganteum*. In our 2 field sites these low temperatures appeared in mid- to late August and infection levels among sentinels and collected larvae dropped correspondingly.

The fungus persisted even when both sites had little or no standing water. At the McAlpine site, water levels less than 10 cm meant that the standing water was restricted to a few isolated pools; with water levels of 7.5 cm or less, only one or two shallow puddles remained of the original 800 m^2 site. The sentinel datum for day 66 (Table 2) in the McAlpine site was obtained in a small puddle that was the last remnant of standing water. Twenty-five percent of these sentinel larvae became infected during their 48 hr exposure; 14% of the larvae exposed to a water sample became infected. When flooding caused the site to be resubmerged and the larval mosquito population to rise, the fungus infected sentinel larvae at all stations in the site. We observed oospores of *L. giganteum* in a large proportion of infected larvae. These oospores may have been responsible for the survival of the fungus.

Establishment of *L. giganteum* in the McAlpine site was remarkable because only 7 petri dishes of fungal culture were introduced into a small portion of the 800 m^2 site. The flood on day 2 not only failed to eliminate the fungus but spread it throughout the site. The implication of these events is that successful introduction of *L. giganteum* into a site may be accomplished by adding the fungus to only one portion and letting it spread.

Glenn and Chapman (1978) reported three years' observations of a natural epizootic of *L. giganteum* in a black gum swamp. During that time the fungus was present in the mosquito population for 9 of 21 months. Infection levels among the weekly collections of larvae ranged from 38 to 100%. The fungus recrudesced after at least two winters during which the mean daily temperatures were 6° to 14°C and after one four-month drought. Interestingly, only *Cx. territans* was infected while associated *Aedes* and *Psorophora* were not infected. In our study, all of the mosquito species previously mentioned as being present were represented by varying numbers of infected individuals in the samples. However, because no quantitative expression of the relative susceptibilities of the species could

be made due to the great variation in sample sizes and species composition, the data for the different species were combined.

In long-term studies in California, Washino (1981) has observed *L. giganteum* persisting at least 8 years in a drainage ditch where it was introduced by McCray et al. (1973). The fungus failed to survive in two other sites. Unfortunately, few detailed data from these studies have been published. Fetter-Lasko (cited in Washino 1981) demonstrated survival of *L. giganteum* in an experimental rice field after one winter during which the site was drained, rice harvested, crop residue burned and the field disked and replanted. In the second year of the trial, infection rates were 19.4 to 48.5% of the groups of sentinel larvae exposed in the rice field plots.

Our data from monitoring *L. giganteum* after a single introduction into upland fresh water sites indicate that the fungus can be a self-perpetuating biocontrol agent of larval mosquitoes for at least an entire mosquito breeding season. *Lagenidium giganteum* may be valuable in controlling or reducing mosquito production in relatively inaccessible habitats where the frequent application of larvicides is impractical, provided of course, the water quality is suitable for survival of the fungus (Axtell et al. 1982). Further studies of *L. giganteum,* after artificial introduction under a variety of conditions, are necessary to determine how well it persists and gives long-term reduction of mosquito populations.

The two sites were sampled for the presence of *L. giganteum* in June 1983, 1 year after the initial introduction of the fungus. Mosquito larvae (*Culex* spp.) collected from both sites were infected (15 out of 91 from the McAlpine site and 10 out of 35 from the New Hope River site). Larvae (second-instar) of laboratory-reared *Cx. quinquefasciatus* which were added to water collected from the McAlpine site became infected (19 out of 75). These data show that the fungus survived overwinter at these sites. However, annual augmentation may still be necessary for effective mosquito control.

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References Cited

American Public Health Assoc. 1976. Standard methods for the examination of water and waste water. 14th ed., APHA, Washington, D.C. 874 p.

Axtell, R. C., S. T. Jaronski and T. L. Merriam. 1982. Efficacy of the mosquito fungal pathogen, *Lagenidium giganteum* (Oomycetes: Lagenidiales). Proc. Calif. Mosq. Vector Control Assoc. 50:41–42.

Christensen, J. B., J. L. Fetter-Lasko, R. K., Washino, R. C. Husbands and E. E. Kaufman. 1977. A preliminary field study employing *Lagenidium giganteum,* a fungus, as a possible biological control agent against the pasture mosquito *Aedes nigromaculis.* Proc. Calif. Mosq. Vector Control Assoc. 45:105 (abstract).

Domnas, A. J., S. M. Fagan and S. T. Jaronski. 1982. Factors influencing zoospore production in liquid cultures of the mosquito pathogen *Lagenidium giganteum* (Oomycetes; Lagenidiales). Mycologia 75:820–825.

Glenn, F. E. and H. E. Chapman. 1978. A natural epizootic of the aquatic fungus *Lagenidium giganteum* in the mosquito *Culex territans.* Mosq. News 38:522–524.

Jaronski, S. T. and R. C. Axtell. 1982. Effects of organic water pollution on the infectivity of the fungus *Lagenidium giganteum* (Oomycetes; Lagenidiales) for mosquito larvae *(Culex quinquefasciatus)*: Field and laboratory evaluation. J. Med. Entomol. 19:255–262.

Jaronski, S. T. and R. C. Axtell. 1983. Effects of temperature on infection growth and zoosporogenesis of *Lagenidium giganteum,* a fungal pathogen of mosquito larvae. Mosq. News 43:42–45.

Jaronski, S. T., R. C. Axtell, S. M. Fagan and A. J. Domnas. 1983. In vitro production of zoospores by the mosquito pathogen *Lagenidium giganteum* (Oomycetes; Lagenidiales) on solid media. J. Invert. Pathol. 41:305–309.

McCray, E. M., D. J. Womeldorf, R. C. Husbands and D. A. Eliason. 1973. Laboratory observations and field tests with *Lagenidium* against California mosquitoes. Proc. Calif. Mosq. Control Assoc. 41:123–128.

Washino, R. K. 1981. Biocontrol of mosquitoes associated with California rice fields with special reference to the recycling of *Lagenidium giganteum* Couch and other microbial agents, pp. 122–139, *In* M. Laird (Ed.)., Biocontrol of medical and veterinary pests. Praeger Publishers, New York.

Washino, R. K. and C. K. Fukushima. 1978. Assessment of biological control agents against mosquito larvae in Northern California: A progress report. Proc. Calif. Mosq. Vector Control Assoc. 46:89 (abstract).