Lagenidium giganteum Zoospores: Effects of Concentration, Movement, Light, and Temperature on Infection of Mosquito Larvae

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Assays conducted with a California isolate of *Lagenidium giganteum* revealed that the LC_{50} against second-instar *Culex quinquefasciatus* larvae in pond water was 26 zoospores/ml. The maximum distance that zoospores swam horizontally and infected second-instar larvae was 60 cm. The maximum depth from which zoospores moved upward in a column of water and infected larvae was between 30 and 40 cm. The release depth of zoospores in a column of water at which 50% larval mortality occurred was 19.6 cm. Free-swimming zoospores did not exhibit positive or negative attraction to incandescent light as evidenced by larval mortality. Survival of zoospores as indicated by mosquito larval mortality was greatest at 25°C and was similar at 30, 33, and 35°C. No infection occurred at 17°C and <20% larval mortality occurred at 19°C for any age of zoospores. © 1999 Academic Press

Key Words: *Lagenidium giganteum*; *Culex quinquefasciatus*; zoospores; behavior; temperature.

INTRODUCTION

Lagenidium giganteum Couch (Oomycetes: Lagenidiales) is a fungal¹ pathogen that has shown great potential as a biocontrol agent for mosquito larvae in unpolluted freshwater habitats (Federici, 1981; Axtell *et al.*, 1982; Jaronski, 1982; McCray, 1985; Lacey and Undeen, 1986; Lacey and Lacey, 1990; Hornby *et al.*, 1992; Cuda *et al.*, 1997). In nature, it may grow vegetatively as a saprophyte or complete its life cycle as a parasite within mosquito larvae (Couch, 1935). The parasitization process is initiated by a biflagellate zoospore (infectious unit) that attaches and encysts on the cuticle of the larva. Zoospores may encyst on the external cuticle and/or may be ingested and encyst on the cuticle-lined foregut. Once encysted, the contents of

the zoospore are expelled into the body cavity of the larva via a germ tube. The contents give rise to vegetative hyphae that proliferate throughout the mosquito larva, particularly around the head and thorax region, and eventually overwhelm and kill the larva.

Aside from the infection process, very little is known about the behavior of *L. giganteum* zoospores. It is apparent that many factors must be recognized in order to maximize the efficacy of *L. giganteum* zoospores against mosquito larvae in natural breeding habitats. A study was initiated to gain a better understanding of zoospore behavior with experiments focused on: (1) zoospore concentration–larval mortality relationships, (2) horizontal and vertical movement of zoospores, (3) phototaxis, and (4) zoospore survival at various temperatures. This information can be used to maximize success of inoculative releases of *L. giganteum* against mosquito larvae in natural breeding habitats.

MATERIALS AND METHODS

Fungus and mosquito cultures. A California isolate of *L. giganteum* was used in all experiments and was obtained in 1987 from J. Kerwin, University of California at Davis. A North Carolina isolate, used only in the zoospore concentration assay, was collected from naturally occurring infected mosquito larvae near Chapel Hill, North Carolina in 1990. Both isolates were maintained and subcultured routinely on a medium of water extract of ground sunflower seed (SFE) as previously described (Jaronski and Axtell, 1984; Guzman and Axtell, 1986). Cultures were grown in 100 ml of medium in 500-ml Erlenmeyer flasks on a rotary shaker (100 rpm) for 7 days at $25 \pm 3^\circ\text{C}$. Aliquots (0.5 ml) of the 7-day-old fungus culture were used to inoculate SFE–agar petri plates (100 × 15 mm), which were incubated at $25 \pm 3^\circ\text{C}$ for 3 days and then stored at $15 \pm 1^\circ\text{C}$ until needed for the experiments. Both isolates were passed through second-instar *Culex quinquefasciatus* Say larvae (20 passes), reisolated, and cultured *in vitro* at about 12-month intervals to maintain virulence.

When zoospores were needed, inoculated plates with

¹ The Oomycetes are now considered to be grouped with the brown algae and the diatoms and should be considered protists (Cavalier-Smith, 1989). However, in this paper we prefer to maintain the fungal designation for *L. giganteum*.

heavy presporangial development were submerged in deionized water ($26 \pm 3^\circ\text{C}$) for 30 min, rinsed thoroughly, and resubmerged in fresh deionized water for 4 h. Plates were given a final rinse and resubmerged with zoospores appearing approximately 12 h after the final rinse.

Larvae of *C. quinquefasciatus* were used in all experiments and were obtained from a laboratory colony established in 1989 from egg rafts collected in the vicinity of Raleigh, NC. Larvae were reared in distilled water and fed dried liver powder.

Pond water. The pond water used in all experiments was collected from a small pond located on the grounds of University Field Laboratories, Raleigh. The water was filtered through a cotton mesh cloth and stored at room temperature for 3 days (to allow sedimentation), at which time the water was siphoned into 3.8-liter plastic jugs and stored in a freezer. When pond water was needed, jugs were removed and thawed for 2 days. Water quality tests were performed (Department of Zoology, North Carolina State University) prior to each experiment; the ranges of values were: pH 6.4 to 6.8; NH_3 (ammonia nitrogen), 0.12 to 0.28 mg/L; TP (total phosphorus), 0.26 to 0.63 mg/L; and COD (chemical oxygen demand), 30 to 40 mg/L.

Zoospore concentration effects. The concentration of *L. giganteum* zoospores in a stock suspension was estimated with a hemacytometer under $10\times$ magnification by taking the average of four readings. Aliquots of the stock suspension containing zoospores (≤ 2 h old) were added to white plastic cups (9 cm diameter $\times$ 5 cm height) containing 100 ml pond water and 20 second-instar *C. quinquefasciatus* larvae to yield final concentrations of 300, 200, 150, 100, 75, 50, 25, 10, 5, and 0 (control) zoospores/ml. The cups were held at $25 \pm 3^\circ\text{C}$ under continuous light for 48 h at which time dead larvae were counted. Two to six dead larvae per cup from each concentration were examined microscopically to verify fungal infection. The experiment was conducted three times using a slightly different set of concentrations for each trial. There were six replicates in the first two trials and eight replicates in the third trial. The data from all three trials were subjected to a generalized linear model using PROC GENMOD (SAS, 1996), which allowed for trial effects and concentration by trial interactions. Since there were no significant trial or interaction effects, probit analysis was done on the combined data set using PROC PROBIT (SAS, 1996).

Horizontal movement. Plastic gutter troughs (6 cm high $\times$ 10 cm wide $\times$ 200 cm or 100 cm long) were filled with pond water to a depth of 4 cm. Ten second-instar larvae, held in cylindrical mesh (70 μm nylon) cages (7 cm diameter $\times$ 6 cm height) were placed at each end of the trough, equidistant from the center of the trough. A previous study (C. Suh, unpublished data) showed that

zoospores could freely pass through the mesh cages. Three-fourths of a SFE-agar plate containing presporangia was placed in the center of the trough just prior to zoospore emergence. The other $\frac{1}{4}$ of the plate was placed in a plastic cup (9 cm diameter $\times$ 5 cm height) containing 100 ml of pond water and 10 second-instar larvae; if zoospores did not produce 100% larval mortality in these cups, the data from the corresponding plate were rejected. The larvae were pipetted from the troughs about 36 h after zoospore emergence and held in pond water for an additional 24 h at which time dead larvae were counted and examined for infection. The control group consisted of four plastic cups each containing 100 ml of pond water and 20 second-instar larvae. The distances tested (between agar plate and larvae) were 0, 20, 40, 60, and 80 cm. The experiment was conducted under continuous light and at $24 \pm 1^\circ\text{C}$. Each distance was replicated 12 or 18 times over time, and mean larval mortality was calculated using PROC MEAN (SAS, 1996). Because we were only interested in determining the maximum distance zoospores could travel horizontally, differences in larval mortality among distances were not statistically analyzed.

Vertical movement. Opaque white plastic polyvinyl chloride (PVC) pipes (10 cm diameter) of various lengths were capped on one end and filled with pond water to heights of 10, 20, 25, 30, and 40 cm. Immediately after the final rinse of the presporangia, $\frac{1}{2}$ of an agar plate was submerged to the bottom of each pipe. Two h after submergence, a nylon mesh (Nitrex cloth, pore opening of 0.0625 mm²) cage (5 cm diameter $\times$ 7 cm height) containing 20 second-instar larvae was placed in each pipe with the bottom of the cage submerged 3 cm under the surface of water. Larvae were removed by pipette after 36 h and held in distilled water for an additional 24 h at which time dead larvae were counted. Two to six dead larvae from each water depth were microscopically examined to confirm that larvae were infected with *L. giganteum*. The control group consisted of four cups, each containing 20 second-instar larvae in 100 ml of pond water. Four pipes were used for each water depth and the experiment was repeated three times to produce a total of 12 replicates for each depth. All experiments were conducted under continuous light and at $28 \pm 1^\circ\text{C}$. The data from all three trials were subjected to a generalized linear model using PROC GENMOD (SAS, 1996), which allowed for trial effects and depth-by-trial interactions. Since there were no significant trial or interaction effects, probit analysis was done on the combined data set using PROC PROBIT (SAS, 1996).

Phototaxis. White plastic troughs (60 cm long $\times$ 10 cm wide $\times$ 6 cm high) were filled to a depth of 4 cm with pond water. Twenty second-instar larvae, contained in mesh cages (described in horizontal movement study), were placed at each end of the trough. Either $\frac{1}{4}$ or $\frac{1}{2}$ of

an agar plate (previously prepared for presporangia) was placed in the center of the trough, and then 1/2 of each trough was covered with black construction paper. All troughs were covered with a sheet of clear plastic to block air currents which could influence zoospore movement. A 15-W incandescent light bulb was suspended 60 cm above the troughs (centered). Forty-eight h after the introduction of the fungus plate into the troughs, the number of dead larvae in each cage was counted. Two to six dead larvae from each cage were examined microscopically to verify *L. giganteum* infection. The control group consisted of three cups each containing 100 ml of pond water and 20 second-instar larvae. The experiment was conducted four times at $26 \pm 2^\circ\text{C}$, with four replicates (troughs) for each trial.

A similar experiment was conducted using Y-shaped troughs constructed from brown plastic troughs (6 cm wide $\times$ 5 cm high). Each of the three arms was 15 cm long and the bifurcation was a 45° angle. The apparatus was filled with pond water to a depth of 3.5 cm. A mesh (70 μm nylon) cage (5 cm diameter $\times$ 4 cm height) containing 20 second-instar larvae was placed at each end of the bifurcation arms. An agar plate containing presporangia was placed at the end of the main straight arm. The entire apparatus was covered with black construction paper except for 6×6 cm at the end of one of the arms containing a cage of mosquito larvae. A single lamp with a 15-W incandescent bulb was suspended 30 cm above the troughs. Dead larvae were counted 54 h after introduction of the agar plates, and two to six dead larvae from each cage were examined microscopically to verify infection. The experiment was conducted three times at 25°C and two times at 30°C with four troughs (replicates) for each trial. Since there were no trial effects, data within each temperature and apparatus type were pooled, and mean larval mortalities compared (*t* test, $P = 0.05$) between light and dark arms using PROC GLM (SAS Institute, 1996).

Temperature effects. White plastic cups (5 cm height $\times$ 8 cm diameter) were each filled with 100 ml of pond water and placed in continuously illuminated (GE Bright Stik 120V 60 Hz) incubators set at constant temperatures (17, 19, 25, 30, 33, and 35°C). After the water reached the respective incubator temperature, 10-ml aliquots of deionized water containing approximately 1.0×10^5 to 1.9×10^5 zoospores (<4 h old) were pipetted into each cup. The number of zoospores/10-ml aliquot was estimated with a hemacytometer under $10\times$ magnification by taking the average of four readings. The cups held at each temperature were divided into six subsets with each cup in each subset receiving 20 mosquito larvae at a specified time interval. The time intervals were 1, 4, 7, 9, 11, and 13 h after introduction of zoospores. For each temperature and time interval, there were two control (no zoospores added) cups each containing 100 ml of water and 20

second-instar larvae. The number of dead larvae in each cup was counted 48 h after introduction of the larvae.

The experiment was conducted over time to yield a total of 9 replicates at 17, 19, 25, and 35°C and 12 replicates at 30 and 33°C . Based on the nature of the experimental design, the data for each temperature were pooled and subjected to log-probit analysis using POLO-PC software (Russell *et al.*, 1977).

RESULTS AND DISCUSSION

Zoospore concentration. In the bioassay system used, a zoospore concentration of 300 zoospores/ml produced 100% larval mortality (Table 1). The LC_{10} , LC_{50} , and LC_{90} values with 95% confidence intervals were 5.3 (4.4–6.2), 26.1 (23.8–28.4), and 127.9 (112.0–148.8) zoospores/ml, respectively. In comparison, a North Carolina isolate (obtained in 1990) of *L. giganteum* was assayed similarly and produced LC_{10} , LC_{50} , and LC_{90} values with 95% confidence intervals of 0.8 (0.4–1.4), 5.0 (3.3–6.7), and 30.0 (25.3–36.2) zoospores/ml, respectively (C. Suh, unpublished data).

Both of the LC_{50} values reported in our study are much lower in comparison to other values previously reported using a North Carolina isolate against *C. quinquefasciatus* larvae (Domnas *et al.*, 1982; Jaronski *et al.*, 1983). However, variances among isolates are to be expected. *L. giganteum*'s ability to infect mosquito larvae has been shown to vary among different isolates (Jaronski and Axtell, 1983; Lord and Roberts, 1985a, 1986). Jaronski (1982) suggested that one possible reason for discrepancies in LC_{50} values of *L. giganteum* zoospores against mosquito larvae may be due to differences in the techniques of handling the treatment concentrations. Perhaps a more important factor is the

TABLE 1

Mean ($\pm$ SE) Percentage Mortality of Second-Instar *Culex quinquefasciatus* Larvae Exposed to Various Concentrations of *Lagenidium giganteum* Zoospores (California Isolate)

Concentration (zoospores/ml)	Replicates	Mean ($\pm$ SE) % larval mortality ^a
300	6	100 (0)
200	8	94.4 (2.2)
150	12	94.6 (1.0)
100	8	85.6 (3.6)
75	12	80.4 (2.4)
50	20	71.8 (2.9)
25	20	40.0 (3.0)
10	20	22.5 (1.8)
5	14	13.6 (2.4)
0 (Control)	20	0 (0)

^a Mean ($\pm$ SE) percent larval mortality 48 h after initial exposure to zoospores; based on 20 larvae/replicate.

type of water (test medium) used in the experiment. We used pond water in our experiment, whereas the previous studies used distilled water. Woodring and Kaya (1992) reported approximately 50% infection for all larval instars of *Culex tarsalis* Coquillett using a concentration of 10 zoospores/ml in rice-field water. Lord and Roberts (1985b), using 3-day-old *Aedes aegypti* (L.) (Athens strain), reported a LC_{50} value of 12.9 zoospores/ml in a 0.2 g/liter peptone medium, 133 zoospores/ml in field water, and 124 zoospores/ml in deionized water. They observed that dilutions of *L. giganteum* zoospores in deionized water caused a rapid decline in zoospore activity, thus reducing infection. They suggested that osmotic stress produced by low solute concentrations somehow inactivated *L. giganteum* zoospores. This decline in zoospore activity has also been observed in other fungal zoospores in distilled water (Gooding and Lucas, 1959; Teakle and Gold, 1964; Ho and Hickman, 1967).

Also, culturing techniques may affect the results of concentration assays. Prolonged rearing on artificial diet can reduce zoospore virulence. In our study, the California and North Carolina isolates were reared similarly, both were passed through mosquito larvae and reisolated yearly to maintain virulence, and both were tested similarly. Yet, LC values differed between the isolates, indicating that some strains of *L. giganteum* may be inherently more virulent than others.

Horizontal movement. Because the distance a zoospore travels depends on how long it remains motile, factors affecting duration of motility also affect the maximum distance the zoospore can travel. Although there are no previous studies concerning horizontal movement of *L. giganteum* zoospores, several studies have shown that addition of certain chemicals prolonged the motility of *L. giganteum* zoospores (Lord and Roberts, 1985a,b) as well as other fungal zoospores (Gooding and Lucas, 1959; Teakle and Gold, 1964). Thus, the chemical nature of the medium in which zoospores occur is very important. The use of pond water in our study was an attempt to simulate natural

TABLE 2

Mean ($\pm$ SE) Number of Second-Instar *Culex quinquefasciatus* Larvae Infected after Exposure to *Lagenidium giganteum* Zoospores Released from Various Horizontal Distances

Distance (cm)	Replicates	Mean ($\pm$ SE) no. infected ^a
20	12	8.7 (0.9)
40	12	4.2 (1.0)
60	18	0.8 (0.4)
80	18	0 (0)

^a Mean ($\pm$ SE) number infected larvae 48 h after initial release of zoospores; based on 10 larvae/replicate.

TABLE 3

Mean ($\pm$ SE) Number Dead Larvae of Second-Instar *Culex quinquefasciatus* Larvae Infected by *Lagenidium giganteum* Zoospores Released from Various Vertical Depths in a Column of Water

Zoospore release depth (cm)	Mean ($\pm$ SE) number dead larvae ^a
10	18.2 (0.5)
20	13.1 (0.9)
25	4.5 (0.3)
30	2.6 (0.6)
40	0 (0)

^a Mean ($\pm$ SE) number of dead larvae 48 h after initial release of zoospores; based on 20 larvae/replicate with 12 replicates for each depth.

mosquito breeding sites. We found that no infection occurred at a distance of 80 cm and only $7.8 \pm 1.8\%$ of larvae were infected at a distance of 60 cm (Table 2). Our results indicate that the maximum horizontal distance traveled by *L. giganteum* with the capability of infecting *C. quinquefasciatus* larvae was approximately 60 cm, which is a much greater distance than for other fungal zoospores reported, e.g., *Phytophthora megasperma* var. *sojae* and *Aphanomyces* sp. (Lockwood and Ballard, 1959; Kuhlman, 1964). Ho and Hickman (1967) found that the zoospores of *P. megasperma* var. *sojae* could swim 56 cm in 1 h, assuming the zoospores swam in a fixed direction and at a constant speed. Kuhlman (1964) found that zoospores of *Phytophthora cinnamomi* could travel a maximum distance of 7.5 cm in soil leachate and 2.5 cm in wet soil. However, it should be noted that under field conditions, the maximum horizontal distance traveled by *L. giganteum* zoospores is greatly influenced by water currents.

Vertical movement. As the height of the water column increased, mortalities of larvae exposed to zoospores released at the bottom of the water column decreased (Table 3). No larval mortality was observed when zoospores were released from a depth of 40 cm, and only $12.9 \pm 10.3\%$ larval mortality occurred at a depth of 30 cm. The zoospore release depth at which 50% larval mortality occurred was 19.6 cm.

Our results are similar to those obtained in a study using alginic acid-encapsulated *L. giganteum* (Rueda et al., 1991), in which infection occurred in a 25-cm column of water but not at a depth of 50 cm. One possible explanation may be that the increased pressure exerted from the column of water at the greater depths affected zoosporogenesis, thereby reducing zoospore production. Gooding and Lucas (1959) reported that the depth of the incubation solution affected the sporangial formation in *Phytophthora parasitica* var. *nicotianae*. However, with the bioassay system used in our study, it is not possible to speculate whether

zoospores were not produced at depths greater than 30 cm or whether zoospores simply could not travel vertical distances greater than 30 cm.

Phototaxis. The mean numbers of dead larvae in the light and dark arms were not significantly different within each test apparatus and temperature condition (t test, $P = 0.05$) (Table 4). This suggests that the zoospores of *L. giganteum* are not attracted to nor repelled by incandescent light. Golkar *et al.* (1993) concluded that *L. giganteum* zoospores did not exhibit positive phototaxis when exposed to a 100-W light at a distance of 50 cm. Laboratory observations during the course of this study as well as from other studies (e.g., Domnas *et al.*, 1982) also support this conclusion. This is not surprising since fungal zoospores exhibiting phototaxis are rare (Robertson, 1972). Most of the zoospores that do exhibit phototaxis are found in the chytrids and a few species of *Allomyces* (Kazama, 1972; Robertson, 1972).

Temperature effects. In our study, zoospore survival was assessed by larval mortality. At 17°C, no larval mortality occurred in pond water when exposed to zoospores, and at 19°C, larval mortality did not exceed 20% regardless of zoospore age (Table 5). When larvae were exposed to 13-h-old zoospores, larval mortality was below 15% in all temperatures tested. Given the concentration of zoospores used in our experiment, the mean maximum age of zoospores with 95% confidence interval capable of producing 50% larval mortality at 25, 30, 33, and 35°C were 7.1 (5.5–9.2), 5.2 (4.1–6.7), 5.2 (3.9–6.6), and 5.9 h (4.4–7.7), respectively.

Concurrently, we conducted the same experiment using deionized water instead of pond water. The use of deionized water, however, resulted in low larval mortalities and variable data (C. Suh, unpublished data). Only 1-h-old zoospores held at 33 and 35°C produced larval mortalities above 50%. The large standard deviations along with erratic low larval mortality indicated that deionized water had adverse effects on zoospore survival and infectivity. The pond water data are more realistic in terms of mosquito breeding habitats and

TABLE 4

Mean ($\pm$ SE) Number of Dead Second-Instar *Culex quinquefasciatus* Larvae Held in Trough Arms Exposed to Light vs Arms Shielded from Light (Dark) When Exposed to *Lagenidium giganteum* Zoospores

Apparatus type	Light ^a	Dark ^a
Y-shaped troughs (25°C)	18.4 $\pm$ 0.4 a	18.2 $\pm$ 0.5 a
Y-shaped troughs (30°C)	13.0 $\pm$ 0.7 a	11.9 $\pm$ 1.0 a
Straight troughs	19.5 $\pm$ 0.4 a	19.9 $\pm$ 0.1 a

Note. Values within a row followed by different letters are significantly different (t test, $P = 0.05$).

^a Mean ($\pm$ SE) number dead larvae out of 20 larvae/replicate.

TABLE 5

Mean ($\pm$ SE)^a Number of Dead *Culex quinquefasciatus* Larvae after Exposure to Various Ages (h) of *Lagenidium giganteum* Zoospores Held at Different Constant Temperatures

Temp (°C) ^a	Age of zoospores (h)					
	1	4	7	9	11	13
19	3.7 (0.9)	2.7 (0.8)	2.3 (0.8)	1.0 (0.4)	0.2 (0.2)	0 (0)
25	17.8 (2.0)	14.5 (2.1)	12.8 (2.4)	10.7 (2.1)	6.3 (1.1)	3.0 (1.0)
30	19.6 (0.2)	16.9 (0.2)	8.1 (1.0)	4.9 (0.6)	2.0 (0.5)	0.3 (0.2)
33	19.8 (0.3)	11.8 (2.4)	9.0 (1.5)	5.9 (1.0)	3.3 (0.7)	0.9 (0.3)
35	18.8 (0.3)	15.0 (0.9)	11.0 (0.9)	7.5 (0.4)	3.0 (0.4)	0.8 (0.3)

^a Regardless of zoospore age, no larval mortality occurred at 17°C.

serve as the basis of our discussion. Jaronski and Axtell (1983) reported that temperatures from 21 to 29°C produced the highest rates of infection by *L. giganteum* in distilled water and that there was a marked decrease in the infection rates above 29 and below 21°C. In our study using pond water, zoospore survival was greatest at 25°C and was similar at 30, 33, and 35°C. We did not find a substantial decrease in the infection rates above 29°C, but there was a marked decrease in the infection rates at 19°C with no larval infection occurring at 17°C. Several differences (e.g., test medium, zoospore age and concentration, and isolate) may be responsible for the discrepancies in our study and other studies. Because we used larval mortality as an indicator of zoospore survival, it is uncertain whether zoospores were unable to survive at the lower temperatures or whether zoospores were alive but unable to infect larvae. This is also the case with the different aged zoospores exposed to larvae.

CONCLUSIONS

The data from our study indicate that for successful mosquito larval control, concentrations of at least 150 zoospores/ml of water, water temperatures between 20 and 35°C, and water depths less than 40 cm are required, using a California isolate of *L. giganteum*.

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