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# Effects of temperature, pH and salinity on the infection of *Leptolegnia chapmanii* Seymour (Peronosporomycetes) in mosquito larvae

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**Abstract**

The effects of temperature, pH, and NaCl concentrations on the infectivity of zoospores of *Leptolegnia chapmanii* (Argentine isolate) were determined for *Aedes aegypti* and *Culex pipiens* under laboratory conditions. Zoospores of *L. chapmanii* were infectious at temperatures between 10 and 35 °C but not at 5 or 40 °C. At the permissive temperatures, mortality rates in young instars were much higher than in older instars and larvae of *Ae. aegypti* were more susceptible to *L. chapmanii* than larvae of *Cx. pipiens*. At 25 °C, *Ae. aegypti* larvae challenged with *L. chapmanii* zoospores resulted in 100% infection at pH levels ranging from 4 to 10. Larvae of *Cx. pipiens* exposed to similar pH and zoospore concentrations resulted in increasing mortality rates from 62% to 99% at pH 4 to 7, respectively, and then decreased to 71% at pH 10. *Aedes aegypti* larvae exposed to *L. chapmanii* zoospores in NaCl concentrations ranging from 0 to 7 parts per thousand (ppt) at 25 °C resulted in 100% mortality while mortality rates for *Cx. pipiens* decreases from 96% in distilled water to 31.5% in water with 6 ppt NaCl. Control *Cx. pipiens* larvae died when exposed at a NaCl concentration of 7 ppt. Vegetative growth of *L. chapmanii* was negatively affected by NaCl concentrations. These results have demonstrated that the Argentinean isolate of *L. chapmanii* tolerated a wide range of temperatures, pH, and salinity, suggesting that it has the potential to adapt to a wide variety of mosquito habitats.


*Keywords:* *Leptolegnia chapmanii*; *Aedes aegypti*; *Culex pipiens*; Temperature; pH; Salinity

## 1. Introduction

Some former oomycetes fungi from the Lagenidiales, now included in the Stramenopiles (Peronosporomycetes) (Adl et al., 2005) have been found to cause natural epizootics in a number of medically important Diptera, especially mosquitoes. The most studied of these pathogens is *Lagenidium giganteum* Couch, which has been the subject of considerable research over the last two decades (Lacey and Lacey, 1990; Scholte et al., 2004). Another important entomogenous fungus from this group, *Leptolegnia chapmanii* Seymour 1977 has been isolated from several mosquito species and found to have a number of interesting biological characteristics (McInnis and Zattau, 1982; Seymour, 1984; Lord and Fukuda, 1988; Fukuda et al., 1997). López Lastra et al. (1999) isolated *L. chapmanii* from the floodwater mosquito *Ochlerotatus albifasciatus* in Argentina which represented the first report of this pathogen from the Southern Hemisphere. López Lastra et al. (2004) determined the susceptibility of 10 species belonging to 5 genera of mosquitoes to this native isolate of *L. chapmanii* under laboratory conditions. However, natural habitats of the mosquito species tested exhibit a wide variety of biotic and abiotic conditions not reflected in the previous laboratory studies which could help explain the limited natural occurrence of this Argentinean isolate of *L. chapmanii*. In this report, we have conducted laboratory tests to determine the effects of temperature, pH, and

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NaCl concentrations on the infectivity of the zoospores of the Argentine isolate of *L. chapmanii* for larvae of the mosquitoes *Aedes aegypti* (L.) and *Culex pipiens* L.

## 2. Materials and methods

### 2.1. Pathogen culture

The Argentinean isolate of *L. chapmanii* (CEP 010, ARSEF 5499) was maintained on Emersońs YpSS agar media (yeast extract 4 g, $HK_2PO_4$ 1 g, $MgSO_4$ 0.5 g, starch 15 g, agar 20 g, distilled water 1000 ml) in $60 \times 15$ mm sterilized Petri dishes. Inoculum was prepared by cutting cubes of agar containing hyphae (0.5 $cm^2$) which were placed in 20 ml of sterile distilled water in $90 \times 15$ mm diameter sterilized Petri dishes. Zoospores were released in the water after approximately 72 h at which time third-instar *Ae. aegypti* were transferred to the Petri dishes. Dead larvae were removed and examined under phase contrast microscopy to confirm fungal infection. *Leptolegnia chapmanii* infected *Ae. aegypti* larvae (48 h post-infection) were used as inoculum. Each *L. chapmanii* infected *Ae. aegypti* larvae (1 larval equivalent or LE) was estimated to contain $6.1 \pm 0.2 \times 10^4$ zoospores ($n = 60$).

### 2.2. Mosquito larvae

The larvae of *Ae. aegypti* and *Cx. pipiens* used in this study were obtained from colonies maintained at Centro de Estudios Parasitológicos y de Vectores—CEPAVE, La Plata, Argentina following standard mosquito rearing techniques (Gerberg et al., 1994).

### 2.3. Temperature

Twenty-five larvae of each instar and pupae of the two mosquito species tested were placed in 200 ml plastic containers containing 100 ml of dechlorinated tap water. Zoospores of *L. chapmanii* equivalent to 3 LE were added to each container. Temperatures of 5, 10, 15, 20, 25, 30, 35, and 40 °C were simultaneously maintained in separate incubators (±0.1 °C) for the experiments. Three containers and one control with larvae and no fungus added were maintained at each temperature tested.

### 2.4. pH

The effect of pH on *L. chapmanii* infection rates was determined in containers by manipulating solutions of Tris–sodium citrate (0.5 M) and Tris–citric acid (0.5 M) to produce pH values ranging from 4 to 10. Twenty-five third or early fourth-instar larvae of *Ae. aegypti* and *Cx. pipiens* were exposed to zoospores of *L. chapmanii* (3 LE) in containers with 100 ml of the above solutions. Three containers and one control with larvae and no fungus added were maintained at each pH value in incubators at 25 °C.

### 2.5. NaCl concentrations

Twenty-five third or early fourth-instar larvae of the two mosquito species tested were exposed to zoospores of *L. chapmanii* (3 LE) in each of the three 200 ml plastic containers and one control with larvae and no fungus added containing 100 ml of dechlorinated water with 0, 1, 2, 3, 4, 5, 6, and 7 parts per thousand (ppt) of NaCl at 25 °C.

Vegetative growth of *L. chapmanii* in artificial media with NaCl added was determined in $60 \times 15$ mm sterilized Petri dishes with Emerson's YpSS agar media and NaCl concentrations from 0 to 30 ppt. A small cube of agar containing hyphae (0.5 $cm^2$) was placed in the center of each of the three Petri dishes used for every NaCl concentration tested and maintained at 25 °C. Fungal growth was measured at 24, 48, 72, and 92 h post-inoculation.

All assays described above (temperature, pH, NaCl concentrations) were replicated three times on separate dates under similar conditions.

### 2.6. Data analysis

Mortality data were converted to percentage, arcsine square-root transformed, and analyzed using a bifactorial model of analysis of variance (ANOVA). The Tukey multiple comparison test was used to determine if significant differences existed between means of instar mortality at different temperatures. Significance of the differences between larval mortality rates at different pH values and different NaCl concentrations were analyzed by the Duncan test.

## 3. Results

### 3.1. Effects of temperature

Significant differences in infectivity with *L. chapmanii* were found between larvae instars and temperatures (ANOVA, $p < 0.0001$). Larvae of *Ae. aegypti* were significantly more susceptible than larvae of *Cx. pipiens* to infection with *L. chapmanii* at all temperatures ($p < 0.05$). Mortality rates in young instars exposed to *L. chapmanii* were much higher than in older instars. The lowest infection rates were found for pupae of *Ae. aegypti* (3.6%) at 35 °C (Table 1) and *Cx. pipiens* (2.2%) at 10 and 35 °C (Table 2). Mortality rates for *Ae. aegypti* (Table 1) and *Cx. pipiens* (Table 2) were significantly affected by larval instar and temperature. As determined by Tukey test, maximum mortality rates for each larval instar occurred at 25 °C for *Ae. aegypti* (Table 1) and at 20 °C for *Cx. pipiens* (Table 2). Larval and pupal mortality was not found for either mosquito species exposed to *L. chapmanii* in containers held at 5 and 40 °C.

### 3.2. Effects of pH

*Aedes aegypti* larvae exposed to *L. chapmanii* zoospores resulted in 100% infection at all pH levels from 4 to 10

Table 1
Percent mortality (means ± SD) of *Aedes aegypti* immature stages infected with *Leptolegnia chapmanii* at different temperatures

| Instar | Temperature | | | | | |
|---|---|---|---|---|---|---|
| | 10 °C | 15 °C | 20 °C | 25 °C | 30 °C | 35 °C |
| I | 100 ± 0a | 100 ± 0a | 100 ± 0a | 100 ± 0a | 100 ± 0a | 87.1 ± 1.7a |
| II | 80.3 ± 1.2b | 100 ± 0a | 100 ± 0a | 100 ± 0a | 99.5 ± 0.1 ab | 82.6 ± 1.2ab |
| III | 42.6 ± 1.2c | 100 ± 0a | 100 ± 0a | 100 ± 0a | 77.3 ± 1.02c | 72.8 ± 1.4bc |
| IV | 37.8 ± 1.3cd | 72.1 ± 1.8b | 96.4 ± 1.8b | 99.1 ± 0.3a | 67.1 ± 2.3cd | 64.4 ± 1.5cd |
| Pupa | 3.9 + 0.5 | 7.1 + 0.8 | 10.6 + 0.8 | 15.5 + 0.9 | 6.6 + 0.4 | 3.5 + 0.4 |

Percent mortality followed by the same letter are not significantly different according to the Tukey test ($\alpha = 0.01$). No mortality was recorded in controls.

Table 2
Percent mortality (means ± SD) of *Culex pipiens* immature stages infected with *Leptolegnia chapmanii* at different temperatures

| Instar | Temperature | | | | | |
|---|---|---|---|---|---|---|
| | 10 °C | 15 °C | 20 °C | 25 °C | 30 °C | 35 °C |
| I | 62.6 ± 3.1a | 100 ± 0a | 100 ± 0a | 100 ± 0a | 72.8 ± 2.6a | 66.6 ± 1.5a |
| II | 37.7 ± 1.1b | 100 ± 0a | 100 ± 0a | 100 ± 0a | 64 ± 2.5ab | 54.6 ± 1.9b |
| III | 26.6 ± 1.6b | 65.3 ± 1.6b | 92.8 ± 1.4a | 70.2 ± 1.4b | 58.2 ± 3abc | 40.9 ± 1.03bc |
| IV | 13.7 ± 1.6c | 48 ± 2.03bc | 84.8 ± 1.3a | 40.4 ± 1.9c | 29.7 ± 2.5bc | 29.7 ± 1.2bc |
| Pupa | 2.2 ± 0.4 | 4.8 ± 0.42c | 5.3 ± 0.8 | 8.9 ± 1.06 | 3.1 ± 0.72 | 2.2 ± 0.4 |

Percent mortality followed by the same letter are not significantly different according to the Tukey test ($\alpha = 0.01$). No mortality was recorded in controls.

while no mortality was recorded in any of the control containers (Fig. 1). Larvae of *Cx. pipiens* exposed to similar pH and zoospore concentrations resulted in an increasing mortality rate of 62% to 99% at pH 4 to 7, respectively; mortality rates decreased to 71% at pH 10 (Fig. 1). Mortality rates of *Cx. pipiens* larvae exposed to *L. chapmanii* zoospores at pH values from 4 to 10 were highly significant (ANOVA, $p < 0.0001$).

### 3.3. Effects of NaCl concentrations

Mortality rates of 100% were obtained for *Ae. aegypti* larvae exposed to *L. chapmanii* zoospores in NaCl concentrations ranging from 0 to 7 ppt. Mortality decreased from 96% in distilled water to 31.5% in water with 6 ppt of NaCl when larvae of *Cx. pipiens* were exposed to *L. chapmanii* (Fig. 2). Control larvae of *Cx. pipiens* could not tolerate NaCl concentrations higher than 6 ppt. Split-plot ANOVA analysis of mortality rates of *Cx. pipiens* larvae by *L. chapmanii* exposed in NaCl concentrations from 0 to 6 ppt were highly significant ($p < 0.0001$).

The results of *in vitro* vegetative growth of *L. chapmanii* indicated that NaCl reduced mycelial growth (Fig. 3) with complete inhibition occurring at 15 ppt and higher during the first 48 h. From day 3 to 4 low levels of mycelial growth were recorded in media with 15 and 20 ppt NaCl while no vegetative growth occurred in media with 30 ppt NaCl (Fig. 3).

## 4. Discussion

The results reported here have demonstrated that the Argentine isolate of *L. chapmanii* is infective for *Ae. aegypti*

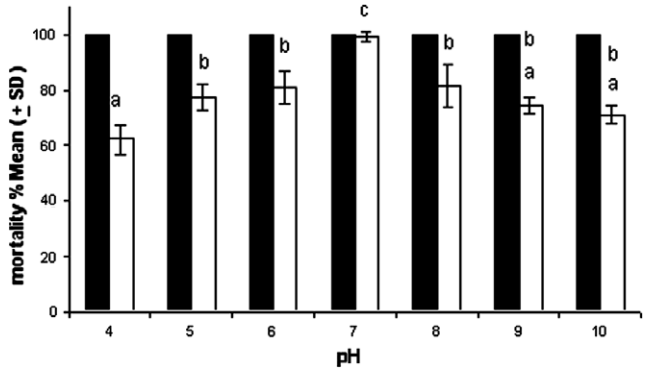


Fig. 1. Percent mortality (means ± SD) of *Aedes aegypti* (dark bars) and *Culex pipiens* (clear bars) larvae exposed to zoospores of *Leptolegnia chapmanii* at different pH regimes. Percent mortality followed by the same letter are not significantly different according to the Duncan test ($\alpha = 0.01$). No mortality was recorded in controls.

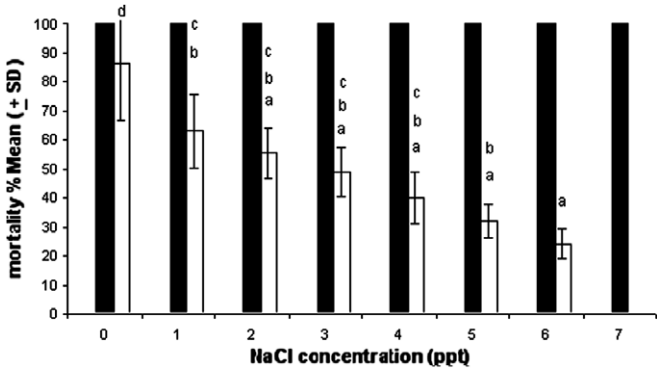


Fig. 2. Effect of NaCl concentrations on the percent mortality (means ± SD) of *Aedes aegypti* (dark bars) and *Culex pipiens* (clear bars) larvae infected with *Leptolegnia chapmanii*. Percent mortality followed by the same letter are not significantly different according to the Duncan test ($\alpha = 0.01$). No mortality was recorded in controls.

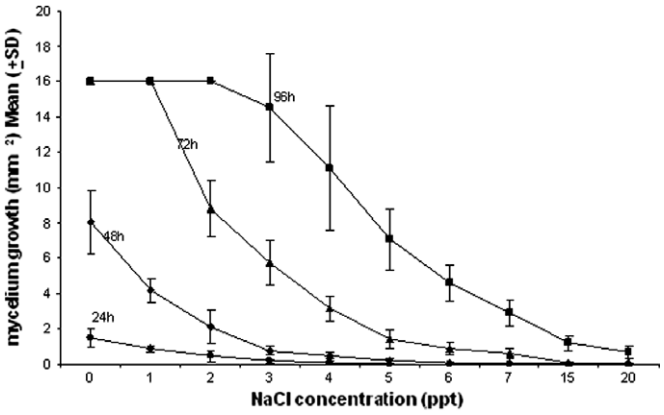


Fig. 3. *In vitro* growth (means ± SD) ($mm^2$) of *Leptolegnia chapmanii* mycelium on Emersońs YpSS agar media containing various concentrations of NaCl. No mortality was recorded in controls.

and *Cx. pipiens* larvae over a wide range of temperatures, pH, and NaCl concentrations. Infections, however, did not occur at temperatures of 5 and 40 °C which are at the tolerance limits of the mosquito hosts. As expected, younger instars of *Ae. aegypti* and *Cx. pipiens* were more susceptible than older instars with pupa being the most refractory stage to infections with *L. chapmanii*. The highest infection rates were found at 20 and 25 °C for all larval instars of the two mosquito species tested.

*Lagenidium giganteum* Couch is the oomycetes that has been most thoroughly investigated over the last two decades. The highest larval infection rates with two isolates of *L. giganteum* (North Carolina and Louisiana isolates) occurred at 21–29 °C for both isolates and there was a marked decrease in infection rates above 29 °C and below 21 °C (Jaronski and Axtell, 1983). These temperatures correspond to the range required for successful zoosporogenesis in *L. giganteum* (Jaronski et al., 1983). Those two isolates of *L. giganteum* showed differences in temperature tolerance suggesting that different strains were adapted to specific habitats.

*Crypticola clavulifera* Humber, Frances and Sweeney is another species of Oomycota reported from a dipteran host. It was isolated from larvae of *Forcipomyia marksae* Tokunaga (Diptera: Ceratopogonidae) in Australia in 1985 and was capable of infecting mosquito larvae (Frances et al., 1989). The biology of *C. clavulifera* is typical of Lagenidiales and similar to *L. giganteum* and *L. chapmanii*. Frances (1991) studied the host range and temperature tolerance of *C. clavulifera* in mosquitoes and found that mosquito infection was inhibited at temperatures of 30 °C and above because the fungus was killed and therefore zoospores were not produced.

The native isolate of *L. chapmanii* studied here can tolerate a broad range of hydrogen ion concentrations (pH) producing high rates of infection in *Ae. aegypti* and *Cx. pipiens* larvae. Mortality rates greater than 60% were recorded from pH 4 to 10 which is within the range of many mosquito larval habitats (MacGregor, 1929). Lord and Roberts (1985) determined that statistically significant zoospores production was recorded for several isolates of *L. giganteum* in artificial media at pH from 4.5 to 8.4. The effect of pH on the infectivity of *L. chapmanii* or *L. giganteum* for larval mosquitoes has not been investigated prior to this study.

Growth of this isolate of *L. chapmanii* was negatively affected by the presence of NaCl in the culture media. Lord et al. (1988) determined that concentrations up to 5 ppt of NaCl enhances mycelial growth of an isolate of *L. chapmanii* from an unidentified *Culex* larva collected from a ground pool at the edge of a salt marsh in FL, USA. Higher concentrations of NaCl were progressively inhibitory, with growth of this Florida isolate failing to occur at 20 ppt. This was similar to the growth rate pattern reported for *L. giganteum* on saline agar (Merriam and Axtell, 1982).

Significant differences in mortality rates between *Ae. aegypti* and *Cx. pipiens* larvae were found in this study when exposed to *L. chapmanii* zoospores in saline water. Saline concentrations did not affect infection rates by *L. chapmanii* in *Ae. aegypti* larvae but infection in larvae of *Cx. pipiens* was significantly affected by the NaCl concentration. The reasons for the difference in the susceptibility of *Ae. aegypti* and *Cx. pipiens* larvae to *L. chapmanii* is not clear.

The viability of *L. chapmanii* zoospores was less affected by high NaCl concentrations than vegetative growth. This is in contrast to *L. giganteum* which cannot infect larvae in water with greater than 1 ppt NaCl (Merriam and Axtell, 1982; Lord and Roberts, 1985). While this might restrict the natural occurrence of *L. giganteum* to freshwater habitats, *L. chapmanii* has demonstrated a tolerance to high salinity which at some mosquito sites can attain levels of up to 20 ppt (Petersen and Chapman, 1970).

This study has demonstrated that the Argentinean isolate of *L. chapmanii* tolerated a wide range of abiotic factors including temperature, pH, and salinity indicating that it could occur in a variety of mosquito habitats. However, the natural distribution of *L. chapmanii* in Argentina is poorly known as it has only been isolated from *Oc. albifasciatus* (used in this study) and *Ae. aegypti* (López Lastra et al., 2004). The *Oc. albifasciatus* habitat from which *L. chapmanii* was isolated is a freshwater/brackish area where epizootics were recorded (López Lastra et al., 1999). The salt tolerance demonstrated by this isolate may reflect an adaptation to the host's habitat which can vary greatly in salinity. Additional isolates of *L. chapmanii* are needed in order to evaluate its ability to adapt to abiotic factors associated with different mosquito habitats.

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