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Impact of short-term temperature challenges on the larvicidal activities of the entomopathogenic watermold *Leptolegnia chapmanii* against *Aedes aegypti*, and development on infected dead larvae

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1 **Impact of short-term temperature challenges on the larvicidal activities of the**
2 **entomopathogenic watermold *Leptolegnia chapmanii* against *Aedes aegypti*, and**
3 **development on infected dead larvae**

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29 ABSTRACT

30 The oomycete *Leptolegnia chapmanii* is among the most promising entomopathogens
31 for biological control of *Aedes aegypti*. This mosquito vector breeds in small water
32 collections, where this aquatic watermold pathogen can face short-term scenarios of
33 challenging high or low temperatures during changing ambient conditions, but it is yet
34 not well understood how extreme temperatures might affect the virulence and recycling
35 capacities of this pathogen. We tested the effect of short-term exposure of encysted *L.*
36 *chapmanii* zoospores (cysts) on *A. aegypti* larvae killed after infection by this pathogen
37 to stressful low or high temperatures on virulence and production of cysts and oogonia,
38 respectively. Cysts were exposed to temperature regimes between -12°C and 40°C for 4,
39 6 or 8 h, and then their infectivity was tested against third instar larvae (L3) at 25°C; in
40 addition, production of cysts and oogonia on L3 killed by infection exposed to the same
41 temperature regimes as well as their larvicidal activity were monitored. Virulence of
42 cysts to larvae and the degree of zoosporogenesis on dead larvae under laboratory
43 conditions were highest at 25°C but were hampered or even blocked after 4 up to 8 h
44 exposure of cysts or dead larvae at both the highest (35°C and 40°C) and the lowest (-
45 12°C) temperatures followed by subsequent incubation at 25°C. The virulence of cysts
46 was less affected by accelerated than by slow thawing from the frozen state. The
47 production of oogonia on dead larvae was stimulated by short-term exposure to freezing
48 temperatures (-12°C and 0°C) or cool temperatures (5°C and 10°C) but was not detected
49 at higher temperatures (25°C to 40°C). These findings emphasize the susceptibility of *L.*
50 *chapmanii* to short-term temperature stresses and underscore its interest as an agent for
51 biocontrol of mosquitoes in the tropics and subtropics, especially *A. aegypti*, that breed
52 preferentially in small volumes of water that are generally protected from direct
53 sunlight.

54

55 *Keywords:* Temperature stress, mosquito, *Saprolegniales*

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57

58 1. Introduction

59 *Leptolegnia chapmanii* (Straminipila: Peronosporomycetes) is an aquatic oomycete
60 entomopathogen that occurs in small stagnant breeding sites and affects *Aedes aegypti*
61 larvae (McInnis & Zattau 1982, Seymour 1984, McInnis 1985, López Lastra et al. 2004,
62 Montalva et al. 2016). This mosquito is the main vector of dengue, chikungunya and
63 Zika fever in the tropics (Mayer et al. 2017). Larvae are infected with the
64 entomopathogen by cysts through the cuticle or after ingestion (Zattau & McInnis
65 1987). Larvae succumb to infection a few hours or days afterwards (Pelizza et al. 2008).
66 After host death *L. chapmanii* produces zoosporangia with asexual mobile zoospores –
67 that encyst after losing their flagelas– and eventually sexual oogonia that after
68 fecundation develop to oospores (Zattau & McInnis 1987, Pelizza et al. 2010).

69 Concepts about the geographical distribution of this oomycete are currently
70 changing. Until recently the only records of *L. chapmanii* were from the southern USA
71 and Argentina (Humber et al. 2014). Recent findings in Central Brazil, however,
72 suggested that *L. chapmanii* may be widely distributed and possibly even common in
73 tropical America (Montalva et al. 2016). These new findings support data about a wide
74 range of the temperature tolerances of this entomopathogen reported from laboratory
75 studies (Pelizza et al. 2007). While this particular entomopathogen was not infective to
76 *A. aegypti* larvae in the laboratory at 5°C, it was infective at permanent exposure to
77 10°C. In fact, the optimal temperature for larvicidal activity of *L. chapmanii* ranges
78 between 20°C and 30°C (Pelizza et al. 2007). Exposure to 35°C reduced virulence but
79 did not prevent cysts from initiating infections; however, at $\geq 40^\circ\text{C}$ larvae were not
80 infected by *L. chapmanii* (Pelizza et al. 2007). Production of oogonia and resistant
81 oospores on dead larvae was stimulated by prolonged unfavorable high temperature up
82 to 40°C and increasingly retarded by lower temperatures (Pelizza et al. 2010).

83 The water temperature in mosquito breeding sites varies constantly according to
84 the seasonal and circadian weather patterns. Depending on climatic conditions, the type
85 and size of the breeding site and its exposure to weather conditions, the temperature of
86 the water can quickly reach high or low levels for both the pathogen and its hosts
87 (Mohammed & Chadee 2011). The times of exposure to any critical temperature
88 stresses can crucially affect the survival, larvicidal activity and reproduction of this and
89 other entomopathogens. Nothing is known yet about activity and survival mechanisms
90 of *L. chapmanii* after temporary exposures to these challenging low or high
91 temperatures. We report here on the virulence of *L. chapmanii* for *A. aegypti* larvae and

92 on the production of encysted zoospores and oogonia on dead larvae after short-term
93 exposures to a range of temperatures between at -12° and 35°C.

94

95 **2. Material and methods**

96 **2.1 Origin, maintenance and preparation of mosquitoes**

97 The colony of *A. aegypti* used here originated from adults collected in 1996 in
98 La Plata, Argentina, and were reared under laboratory conditions at $27 \pm 1^\circ\text{C}$, 75%
99 relative humidity and 12 h photophase as described by Gerberg et al. (1994). Female
100 adults were fed twice a week on chicken blood, and both female and male adults were
101 able to feed continuously on raisins for their supply with carbon sources. The larvae
102 were fed on small amounts of dry, ground rabbit food pellets (La Tahona, Cerealera
103 Azul, Argentina).

104 Immediately before the tests, recently molted (≤ 12 h) third instar larvae (L3)
105 were separated, transferred twice and kept in distilled sterile water (25 ml) for 1 min and
106 then, without delay, used in the assays.

107

108 **2.2 Origin and maintenance of the pathogen**

109 *Leptolegnia chapmanii* CEP 010 (Collection of Entomopathogenic Fungi of
110 Insects and other Arthropods, CEPAVE) also deposited as ARSEF 5499 in the USDA-
111 ARS Collection of Entomopathogenic Fungal Cultures (ARSEF; Ithaca, New York,
112 USA), was tested. This strain was originally isolated in 1996 from a fourth instar
113 *Ochlerotatus albifasciatus* larva (L4) collected close to the city of La Plata, Argentina
114 (López Lastra et al. 1999). The oomycete was cultivated routinely on Emerson's YPSS
115 medium (yeast extract 4 g, KH_2PO_4 1 g, MgSO_4 0.5 g, starch 15 g, agar 20 g, distilled
116 water 1000 ml) in Petri dishes (50 x 15 mm) at $25 \pm 1^\circ\text{C}$ and a 12 h photophase with
117 weekly inoculation on new medium. The larvicidal activity was maintained by
118 periodical passage through laboratory-reared mosquito larvae, and the pathogen was
119 subsequently reisolated from the mycotized dead larvae (Pelizza et al. 2008).

120

121 **2.3 Production and preparation of encysted zoospores**

122 Mycelium was produced on solid sunflower seed extract (SFE) medium at $25 \pm$
123 1°C and a 12 h photophase for 7 days. For the SFE medium, shelled, unroasted
124 sunflower (*Helianthus annuus*) seeds (100 g) were blended for 2 min, mixed with 1000
125 ml distilled water, the suspension blended for another 2 min, and then filtered through

126 cheese cloth. The residue was mixed in another 1000 ml water for 2 min and filtered
127 again with cheese cloth. Agar (15 g each 1000 ml) was then added to the extract (diluted
128 $\frac{1}{4}$ in distilled water), and the medium was autoclaved (Jaronski et al. 1983). Ten cubes
129 (about 1 cm³ each) of SFE medium with a 7-day culture of mycelium were submerged
130 in 500 ml previously sterilized distilled water in an Erlenmeyer flask and incubated for
131 72 h at $25 \pm 1^\circ\text{C}$ and 12 h photophase. During this period both zoosporogenesis and the
132 encystment of the zoospores occurred (Rueda-Páramo et al. 2015). The water with the
133 cubes and encysted zoospores (hereafter referred as cysts) was then gently agitated by
134 swirling the flasks manually for 1 min. Subsequently, the liquid with the cubes and
135 suspended cysts was filtered through hydrophilic cotton, and the number of cysts in the
136 suspension was determined with a hemocytometer (Neubauer Hemacytometer; Hausser
137 Scientific, Horsham, USA). Cysts maintained in water were used for the tests in the
138 following 24 h. For this, 45 ml of suspended cysts at a final concentration of 2×10^3
139 cysts/ml or 45 ml water only (negative control) were arranged in 50 ml Falcon tubes.
140

141 **2.4 Production and preparation of larvae killed by infection**

142 About 50 L3 prepared as mentioned were exposed to cysts (2×10^3 cysts/ml)
143 suspended in 45 ml distilled water and exposed to $25 \pm 1^\circ\text{C}$ and 12 h photophase.
144 Larvae killed by *L. chapmanii* in the next 24 h were used immediately for the tests. At
145 this time, dead larvae generally were filled with mycelium but had not formed external
146 zoosporangia or zoospores; the presence of mycelium was confirmed by light
147 microscopy (Olympus BX41, Buenos Aires, Argentina). Dead larvae were set
148 individually in plastic Falcon tubes (120 x 15 mm) with 5 ml sterile distilled water each,
149 and the tubes then exposed to initial temperature regimes presented below.
150

151 **2.5 Assays of mosquito responses to simulation of temperature conditions and** 152 **assays**

153 Tubes with suspended cysts, water (control) or dead larvae were exposed for 4 h,
154 6 h or 8 h to defined high or low temperature and then transferred to $25 \pm 1^\circ\text{C}$ until the
155 end of the experiment (maximal 72 h exposure). Conditions of defined initial
156 temperature regimes were provided in a water bath (Masson Digital, Vicking, Buenos
157 Aires, Argentina) at $25 \pm 1^\circ\text{C}$ up to $40 \pm 1^\circ\text{C}$, incubator (Ingelab I-291PF, Buenos
158 Aires, Argentina) at $10 \pm 1^\circ\text{C}$, refrigerator (White-Westinghouse WW-234, Buenos
159 Aires, Argentina) at $5 \pm 1^\circ\text{C}$ and $0 \pm 1^\circ\text{C}$ (ice bath), and in a freezer (Gafa Eurosystems

160 360, Frimetal, Rosario, Argentina) at $-12 \pm 1^\circ\text{C}$. Samples kept at -12°C were thawed
161 either quickly (within 20 min) in a water bath at $25 \pm 1^\circ\text{C}$ or slowly overnight at $5 \pm$
162 1°C and then kept as mentioned. Temperatures at -12°C were monitored routinely with
163 a mercury-in-glass thermometer (Incoterm, Hongkong, China), 0°C in the ice bath with
164 a digital thermometer and higher temperatures up to 40°C were registered also digitally
165 in the water bath. Temperatures generally did not vary by more than 1°C from the set
166 temperature, and in order to simplify the presentation of tested temperatures the 1°C
167 variation is not presented in the following.

168 Twenty healthy L3 were added to each tube with cysts only or water (control),
169 and tubes maintained at 25°C and 12 h photophase for 72 h without feeding the larvae.
170 Larval mortality was monitored for up to 72 h. Dead larvae were retrieved and checked
171 for infection with the Olympus BX41 light microscope.

172 Tubes with dead, mycotized larvae were maintained at 25°C up to 72 h, and the
173 total numbers of suspended cysts from each larva after manual agitation of the tube for
174 about 60 sec were determined at a 24 h, 48 h and 72 h exposure using the light
175 microscope and hemocytometer. The larvicidal activity of these cysts was then checked
176 by exposing 10 L3 prepared as mentioned above in each tube with a dead larva and
177 cysts. Mortality in these second batches of larvae was assessed at a 24, 48 and 72 h
178 exposure, and dead larvae checked for infection as noted above.

179 All tests were run with four independent repetitions, with three replicates (cysts)
180 and four replicates (mycotized larvae with cysts) for each repetition. Percent mortalities
181 were arcsine-square root transformed and then analyzed with analysis of variance and
182 the Student-Newman-Keuls multiple range test for comparison of means. Means were
183 considered to be statistically different at $P < 0.05$. Lethal times to kill 50 and 90% (LT_{50}
184 and LT_{90}) of larvae and their respective confidence intervals (CI) were calculated by
185 probit analysis for dependent data, respectively (Throne et al. 1995).

186

187 **3. Results**

188 **3.1 Larvicidal activity of cysts exposed to different temperatures**

189 Cumulative mortality of larvae was highest (100%) when tested with cysts
190 previously incubated at 25°C (positive control at 0 h) after a 72 h exposure of cysts
191 (Figs. 1a,b, 2). Mortality dropped significantly with increasing exposure periods (up to 8
192 h) of cysts to -12°C (cysts defrosted in a water bath at 25°C : 0 h > 4–8 h or overnight at
193 5°C : 0 h > 4 h > 6 and 8 h; Fig. 1a,b); 0°C ; 35°C and 40°C ($F_{3,92} \geq 12.6$; $P < 0.001$) but

194 not at other temperatures tested (5°C; 10°C and 30°C; $F_{3,92} \leq 2.6$; $P > 0.05$; Fig. 2). At
195 the same time (72 h exposure) there was a highly significant effect of temperature on
196 larval mortality regardless of the exposure period ($F_{7,86} = 50.3$; $P < 0.001$: 0–30°C >
197 35°C and -12°C with cysts thawed in a water bath at 25°C > -12°C with cysts thawed
198 overnight at 5°C > 40°C). Quick and slow thawing procedures had a significant effect
199 on cumulative larval mortality, 72 h after exposure of larvae to cysts regardless of the
200 exposure period to -12°C ($F_{2,18} = 3.7$; $P = 0.04$; water bath at 25°C > overnight at 5°C).
201 Mortality of larvae that were not treated with cysts (negative control) did not exceed
202 8.4% during the same period tested.

203 The lethal times to kill 50% or 90% of the larvae did not differ significantly
204 among the exposure periods (4–8 h) at the same temperature but were significantly
205 different during the same period at different temperatures (Table 1). Values were
206 longest (≥ 36.2 h for LT_{50} and ≥ 66.4 h for LT_{90}) or could not be calculated due to low
207 mortality at the lowest (-12°C) and highest (35°C and 40°C) temperature to which cysts
208 were exposed prior to larval treatment. The shortest values of LT_{50} (≤ 10.3 h) and LT_{90}
209 (≤ 20.4 h) were found at 30°C, followed by the positive control at 25°C (LT_{50} 15.9 h
210 and LT_{90} 26.2 h; Table 1).

211

212 **3.2 Effect of temperature on the development of zoosporangia, oogonia and** 213 **production of encysted zoospores on dead larvae**

214 The largest mean number of cysts/larva ($1.53 \times 10^4 \pm 1.3 \times 10^3$) with maximal 1.8
215 $\times 10^4$ and minimal 1.2×10^4 cysts/larva was produced on dead larvae kept permanently
216 at 25°C for 72 h (positive control; Fig. 3). The significant effect of the exposure period
217 (0 up to 8 h) on quantitative production of cysts from dead larvae increased at higher
218 and lower temperatures tested and was highest at -12°C ($F_{3,44} = 60.7$; $P < 0.001$) –with
219 no detectable zoosporangia, zoospores, or cysts formed at all at this temperature–,
220 regardless of the exposure time (Fig. 3) and thawing technique (not shown in Fig. 3). At
221 the other temperatures tested, zoosporangia, zoospores, and cysts were detected. The
222 numbers of cysts/larva were generally highest between 5°C and 30°C ($\geq 1.26 \times 10^3$
223 cysts/larva), and mostly decreased with longer exposure periods of larvae at test
224 temperatures; no significant difference was found among the values obtained for 4 up to
225 8 h of exposure (Fig. 3).

226 After a 4–6 h exposure of larvae at -12°C, regardless of the thawing technique, \leq
227 62.5% of the larvae formed oogonia. No oogonia were produced after 8 h at this

228 subfreezing temperature. The highest numbers of larvae with oogonia ($\geq 62.5\%$) were
229 found at initial 4–8 h at 0°C . The percentage of larvae with oogonia diminished at
230 higher temperatures (5 and 10°C) and varied between 25% and 43% (at 5°C) and
231 between 12.5% and 25% (at 10°C) without any significant effect of exposure time on
232 the number of larvae with oogonia formed ($F_{2,9} \leq 1$; $P \geq 0.4$). At higher temperatures
233 (25°C up to 40°C), again no oogonia were detected on dead larvae (Table 2). The
234 mortality of healthy larvae newly exposed to dead larvae without oogonia started at a 12
235 h exposure regardless of the initial temperature (-12°C up to 40°C) and exposure time
236 (4–8 h) tested, and reached the highest level (98.9%) at $25\text{--}30^{\circ}\text{C}$ after 72 h (Table 2).
237 Control mortality was $\leq 5\%$ for this test.

238

239 4. Discussion

240 Our results made clear that under the conditions tested, the encysted zoospores
241 of *L. chapmanii* best maintained their virulence and produced the most new zoospores
242 at the continuous exposure time of 3 days at 25°C . Mean temperatures of 25°C are well-
243 known to be optimal conditions for the development and insecticidal activity of *L.*
244 *chapmanii* (Pelizza et al. 2007), as well as for entomopathogenic oomycetes (Jaronski &
245 Axtell 1983, 1984, Frances 1991) and other entomopathogenic fungi (Ferron et al.
246 1991, Croos & Bidochka 1999, Scholte et al. 2004, Fernandes et al. 2008, Maiara et al.
247 2011). However, both larvicidal activity and quantitative zoosporogenesis were
248 hampered or even blocked by a short-term exposure of cyst suspensions to either
249 elevated (35°C and 40°C) or freezing temperatures. Exposure of cysts to increasing
250 periods (4–8 h) at challenging temperature was critical for a larvicidal outcome at both
251 the lowest (-12°C) and highest (35°C and 40°C) temperatures tested but had no real
252 relevance at temperatures between 0°C and 30°C . Challenging temperature shifts can
253 induce entomopathogens to develop resistant structures such as resting spores or other
254 thick-walled, environmentally resistant spore forms (Pelizza et al. 2010, Zhou & Feng
255 2010). The production of oogonia was clearly stimulated in *L. chapmanii* by short
256 exposures of larvae killed previously by this pathogen to freezing (-12°C and 0°C).
257 Stimulation was less evident after short exposure to higher temperatures (5°C – 10°C),
258 and not found at all from 25°C up to 40°C . Oogonia were very rarely produced at 24°C
259 by the *Leptolegnia* strains collected in tropical central Brazil (Montalva et al. 2016).
260 After a prolonged exposure at a 5°C – 40°C range, the minimal time for the appearance

261 of oogonia of this pathogen on *Aedes aegypti* larvae was longest (36 days) at 5°C and
262 shortest at 40°C (5 days) (Pelizza et al. 2010).

263 Both scenarios about the effects of short-term exposure to extreme high or low
264 temperature could be expected for small mosquito breeding sites with low water
265 volumes that are frequently used by *A. aegypti* (Varejão et al. 2005). Smaller water
266 collections adjust more rapidly to changing condition of ambient temperature than do
267 larger collections of water with their slower responses to changing temperatures than to
268 their larger heat capacities. Challenging peaks of short-term high temperature exposures
269 can be expected in regions with tropical or subtropical climate and in regions with
270 temperate or subtropical climates. Peaks of short-term, distinctly low temperatures can
271 be expected during colder periods of the year especially at night in mountainous
272 regions.

273 The accelerated thawing of cyst suspensions at 25°C was more crucial for a
274 higher virulence than was the slower thaw at lower temperatures. The viability and
275 virulence of entomopathogens are better preserved by a reduced ice crystal formation
276 during appropriate selected freezing processes and rapid thawing processes (López
277 Lastra et al. 2002, Delalibera et al. 2004, Humber 2012). The damage to cells stressed
278 by challenging conditions of low or high temperatures is initially reversible and then
279 with increasing exposure becomes irreversible (Roberts & Campbell 1977, Mazur 1984,
280 Benz 1987, Glare & Milner 1991). Sub-freezing temperatures are highly deleterious for
281 cellular survival if the water in the cells freezes in the crystalline (icy) state of water in
282 the cells during either the freezing or thawing processes (Humber 2012).

283 Whereas the aquatic stages of *A. aegypti* seem easily to resist prolonged
284 exposure at challenging low (10°C) or high temperatures (35°C), they did not survive
285 the lowest and highest temperatures (5°C and 40°C, respectively) tested by Pelizza et al.
286 (2007). In another study, larvae of *A. aegypti* survived up to a week at 12°C, and 2.7
287 days at 40°C without developing to pupae (Carrington et al. 2013).

288 The aquatic stages of this mosquito obviously cannot survive either freezing or
289 overheated conditions in breeding sites even after short exposure periods, but those sites
290 may be expected to be repopulated quickly by other individuals developing nearby in
291 larger breeding sites or smaller sites that are better protected against low or high
292 temperatures. New larvae in these sites may be infected by cysts that survived the more
293 extreme temperatures that killed the previous populations of susceptible larvae.

294 *L. chapmanii* was obviously able to produce cryptic infective units on infected
295 larvae challenged previously by freezing temperatures (without any microscopically
296 detectable zoospores, cysts, oogonia or oospores) as new healthy larvae exposed to
297 these cadavers succumbed to infection with this pathogen. These results emphasize the
298 high virulence of this isolate to *A. aegypti* larvae.

299 Knowledge about the potential of *L. chapmanii* for the biological control of *A.*
300 *aegypti* and other mosquitoes is still evolving, and there is no information about the
301 activity of *L. chapmanii* against *A. aegypti* under field conditions (Gutierrez et al. 2017).
302 It is not yet possible to draw any definitive conclusions about the practical utility of this
303 remarkable pathogen in natural settings of this vector. Highly localized application
304 techniques in natural or man-made breeding sites or trap devices protected from short-
305 term peaks of high temperature seems a promising approach for more practical
306 biological control purposes. Recently, *L. chapmanii* was shown to occur also in the
307 tropics (Montalva et al. 2016), and eventually other strains from regions with tropical or
308 sub-tropical climate are better adapted to challenging high temperatures and may be
309 more suitable for the control of *A. aegypti*. The findings of this study about the
310 susceptibility of this pathogen to challenging temperature should strengthen the interest
311 in this entomopathogen as a candidate for the control of *A. aegypti* larvae.

312

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433 Fig. 1. Cumulative mean mortality (%) of *Aedes aegypti* third instar larvae at 25°C after
434 exposure to 2×10^3 *Leptolegnia chapmanii* encysted zoospores/ml for up to 72 h. Cysts
435 were previously incubated at -12°C up to 8 h and then defrosted either in a water bath at
436 25°C for 30 min (a) or overnight at 5°C (b).

437

438 Fig. 2. Cumulative mean mortality (%) (\pm standard error of the mean) of *Aedes aegypti*
439 third instar larvae, after a 72 h exposure to *Leptolegnia chapmanii* encysted zoospores
440 (2×10^3 cysts/ml) or water (negative control) at 25°C. Cysts were previously incubated
441 at 0°C up to 40°C for 0–8 h. Means within the same temperature followed by different
442 letters (a–c) are significantly different ($P < 0.05$) according to the SNK test (without
443 negative control).

444

445 Fig. 3. Mean number (\pm standard error of the mean) of *Leptolegnia chapmanii* encysted
446 zoospores produced for a *Aedes aegypti* third instar larva after a 72 h exposure at 25°C.
447 These larvae were killed previously by the infection (exposure to 2×10^3 cysts/ml), kept
448 at 25°C up to 24 h after death, then exposed to -12°C, 0°C, 10°C, 30°C, 35°C or 40°C
449 after total exposure times at each temperature from 0–8 h. Means within the same
450 temperature followed by different letters (a, b) are significantly different ($P < 0.05$)
451 according to the SNK test.

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Table 1 - Lethal time (hours) to kill 50 or 90% (LT₅₀ and LT₉₀) with their respective confidence interval (CI) and slope \pm standard error of the mean (SE) of *Aedes aegypti* third instar larvae (L3) exposed to water-suspended *Leptolegnia chapmanii* encysted zoospores (2×10^3 cysts/ml) previously exposed at -12°C, 0°C, 5°C, 10°C, 25°C, 30°C, 35°C or 40°C for 4 h, 6 h or 8 h.

Temperature (°C)	Exposure (hours)	Lethal time and CI (hours)		Slope \pm SE
		LT ₅₀	LT ₉₀	
-12*	4	43.2 (14.5–72.8)bc	77.6 (55–165)b	0.04 \pm 0.001
	6	45.1 (17.5–75.3)b	97.3 (66.2–201.2)b	0.02 \pm 0.001
	8	54.2 (39.3–71.7)b	88 (70.8–127.6)b	0.04 \pm 0.001
-12**	4	36.2 (14.8–64.6)bc	66 (47.1–173.8)b	0.04 \pm 0.001
	6–8	***	***	-
0	4	28.2 (19–36.4)bc	56.3 (46.7–72)b	0.05 \pm 0.001
	6	26.7 (9.4–48.6)ab	52.6 (36–142.5)b	0.05 \pm 0.001
	8	26.5 (15.6–94.6)b	62.2 (33–461.4)b	0.04 \pm 0.001
5	4	24.6 (16.4–68.1)bc	61 (33.6–563.5)b	0.03 \pm 0.001
	6	23.4 (12–58.8)ab	58 (33–334)b	0.04 \pm 0.001
	8	25.8 (14.8–70.3)b	64.1 (35.8–497)b	0.03 \pm 0.001
10	4	20.2 (4.3–32.3)ab	41.9 (30.3–76.2)b	0.06 \pm 0.001
	6	22.1 (9.1–36.7)ab	44.8 (31.6–94.2)b	0.06 \pm 0.001
	8	23.7 (10.1–68.8)ab	59.2 (32–842.4)b	0.04 \pm 0.001
30	4	8.2 (2.3–12)a	20.2 (16.6–27.6)a	0.1 \pm 0.03
	6	10.1 (6–12.4)a	17.8 (15.4–22)a	0.16 \pm 0.04
	8	10.3 (5.3–13.2)a	20.4 (17.3–26.2)a	0.12 \pm 0.02
35	4	51.6 (34–81.1)c	172.2 (110–365.5)c	0.06 \pm 0.001
	6–8	***	***	-
40	4–8	***	***	-

Larvae (20 L3 of 4 repetitions each) treated with suspended encysted *Leptolegnia chapmanii* zoospores were kept at $25 \pm 1^\circ\text{C}$, values in the same column of different temperatures followed by different letters (a–c) were significantly different based on the values of CI; negative cumulative control mortality $\leq 8.4\%$ at 25°C at 72 h; LT₅₀ and LT₉₀

of positive control 15.9 (10.6–20.7) h and 26.2 (21.3–36.9) h, respectively (slope \pm SE 0.12 ± 0.01) at 25°C.

* suspended cysts defrosted in a water bath at 25°C for 30 min; ** cysts defrosted overnight at 4°C; *** values of mortality insufficient to calculate lethal times

Table 2 - Relative mean number of dead *Aedes aegypti* third instar larvae (L3) \pm standard error of the mean (SE) with oogonia of *Leptolegnia chapmanii* formed after exposure at different temperature (-12°C up to 40°C) and exposure time (4–8 h) and cumulative mortality of new L3 exposed to dead larvae for 72 h. *

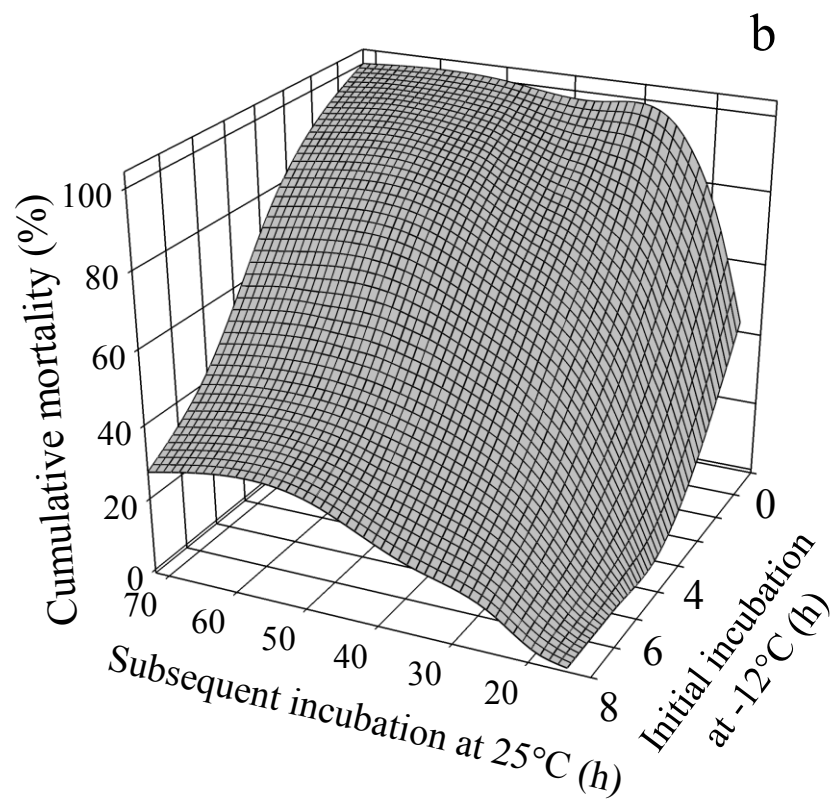
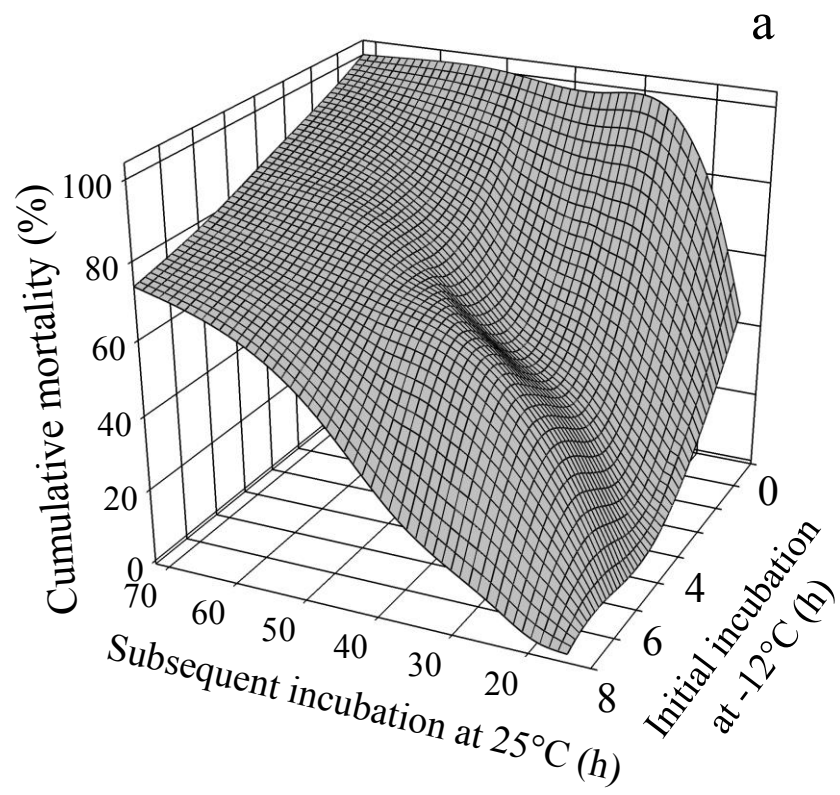
Temperature (°C)	Hours	Percentage of larvae with oogonia (\pm SE)	Mortality % (\pm SE) ****
-12**	4	25 \pm 10.2	45 \pm 15.5
	6	37.5 \pm 16.1	41.3 \pm 20.8
	8	0	19.4 \pm 5.6
-12***	4	12.5 \pm 14.4	70.6 \pm 13.4
	6	62.5 \pm 16.1	27.5 \pm 9.8
	8	0	43.3 \pm 16.9
0	4	62.5 \pm 12.5	73 \pm 23
	6	75 \pm 10.2	90.6 \pm 6.5
	8	68.8 \pm 6.3	87.5 \pm 7.5
5	4	43.8 \pm 12	94.4 \pm 2.8
	6	25 \pm 10.2	91.9 \pm 4.9
	8	25 \pm 10.2	83.1 \pm 11.2
10	4	12.5 \pm 7.2	97.5 \pm 1.4
	6	18.8 \pm 12	92.5 \pm 4.3
	8	25 \pm 10.2	89.4 \pm 7.7
25–30	4–8	0	\geq 98.9 \pm 1.3
35	4–8	0	\geq 48.8 \pm 5.2
40	4–8	0	\geq 41.2 \pm 11.9

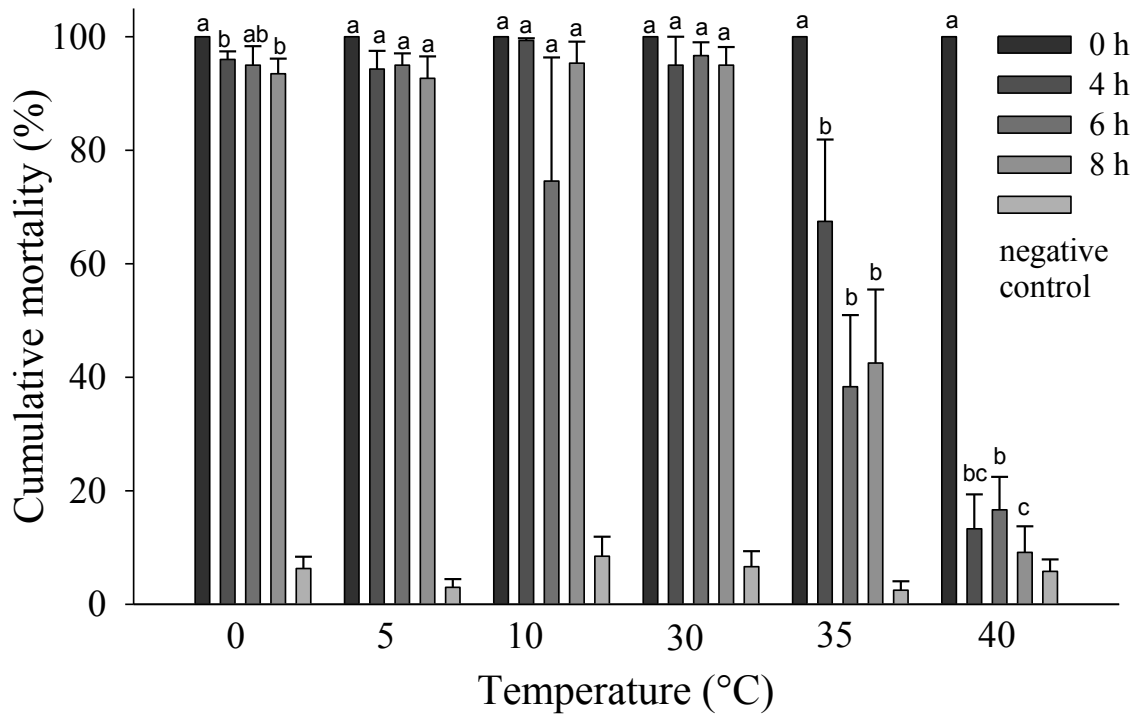
* calculated for a total of 16 L3 each mean value

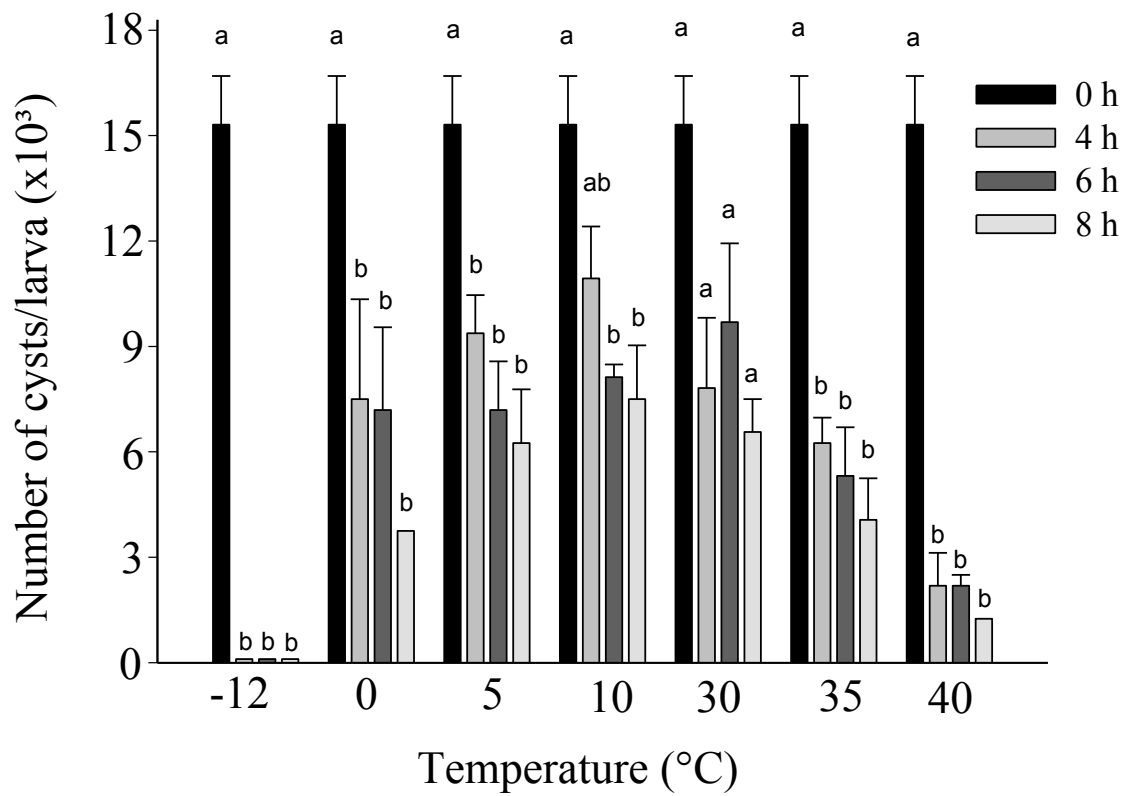
** defrosted in a water bath at 25°C

*** defrosted overnight at 4°C

**** cumulated mean control mortality 0.68%







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