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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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ABSTRACT

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

Keywords: Temperature stress, mosquito, *Saprolegniales*

1. Introduction

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

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

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

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

2. Material and methods

2.1 Origin, maintenance and preparation of mosquitoes

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

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

2.2 Origin and maintenance of the pathogen

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

2.3 Production and preparation of encysted zoospores

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

cheese cloth. The residue was mixed in another 1000 ml water for 2 min and filtered again with cheese cloth. Agar (15 g each 1000 ml) was then added to the extract (diluted ¼ in distilled water), and the medium was autoclaved (Jaronski et al. 1983). Ten cubes (about 1 cm^3 each) of SFE medium with a 7-day culture of mycelium were submerged in 500 ml previously sterilized distilled water in an Erlenmeyer flask and incubated for 72 h at 25 ± 1°C and 12 h photophase. During this period both zoosporogenesis and the encystment of the zoospores occurred (Rueda-Páramo et al. 2015). The water with the cubes and encysted zoospores (hereafter referred as cysts) was then gently agitated by swirling the flasks manually for 1 min. Subsequently, the liquid with the cubes and suspended cysts was filtered through hydrophilic cotton, and the number of cysts in the suspension was determined with a hemocytometer (Neubauer Hemacytometer; Hausser Scientific, Horsham, USA). Cysts maintained in water were used for the tests in the following 24 h. For this, 45 ml of suspended cysts at a final concentration of 2 x 10^3 cysts/ml or 45 ml water only (negative control) were arranged in 50 ml Falcon tubes.

2.4 Production and preparation of larvae killed by infection

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

2.5 Assays of mosquito responses to simulation of temperature conditions and assays

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

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

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

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

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

3. Results

3.1 Larvicidal activity of cysts exposed to different temperatures

Cumulative mortality of larvae was highest (100%) when tested with cysts previously incubated at 25°C (positive control at 0 h) after a 72 h exposure of cysts (Figs. 1ab, 2). Mortality dropped significantly with increasing exposure periods (up to 8 h) of cysts to -12°C (cysts defrosted in a water bath at 25°C: 0 h > 4–8 h or overnight at 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

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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429

430

431

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

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

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

Table 1 - Lethal time (hours) to kill 50 or 90% (LT_{50} and LT_{90}) with their respective confidence interval (CI) and slope ± standard error of the mean (SE) of *Aedes aegypti* third instar larvae (L3) exposed to water-suspended *Leptolegnia chapmanii* encysted zoospores (2 x 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 ± SE
		LT_{50}	LT_{90}	
-12*	4	43.2 (14.5−72.8)bc	77.6 (55−165)b	0.04 ± 0.001
	6	45.1 (17.5−75.3)b	97.3 (66.2−201.2)b	0.02 ± 0.001
	8	54.2 (39.3−71.7)b	88 (70.8−127.6)b	0.04 ± 0.001
-12**	4	36.2 (14.8−64.6)bc	66 (47.1−173.8)b	0. 04 ± 0.001
	6−8	***	***	-
0	4	28.2 (19−36.4)bc	56.3 (46.7–72)b	0.05 ± 0.001
	6	26.7 (9.4−48.6)ab	52.6 (36−142.5)b	0.05 ± 0.001
	8	26.5 (15.6−94.6)b	62.2 (33−461.4)b	0.04 ± 0.001
5	4	24.6 (16.4−68.1)bc	61 (33.6−563.5)b	0.03 ± 0.001
	6	23.4 (12−58.8)ab	58 (33–334)b	0.04 ± 0.001
	8	25.8 (14.8−70.3)b	64.1 (35.8–497)b	0.03 ± 0.001
10	4	20.2 (4.3−32.3)ab	41.9 (30.3−76.2)b	0.06 ± 0.001
	6	22.1 (9.1−36.7)ab	44.8 (31.6−94.2)b	0.06 ± 0.001
	8	23.7 (10.1−68.8)ab	59.2 (32−842.4)b	0.04 ± 0.001
30	4	8.2 (2.3–12)a	20.2 (16.6−27.6)a	0.1 ± 0.03
	6	10.1 (6−12.4)a	17.8 (15.4–22)a	0.16 ± 0.04
	8	10.3 (5.3−13.2)a	20.4 (17.3−26.2)a	0.12 ± 0.02
35	4	51.6 (34−81.1)c	172.2 (110−365.5)c	0.06 ± 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 ± 1ºC, 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 ≤ 8.4% at 25ºC at 72 h; LT_{50} and LT_{90}

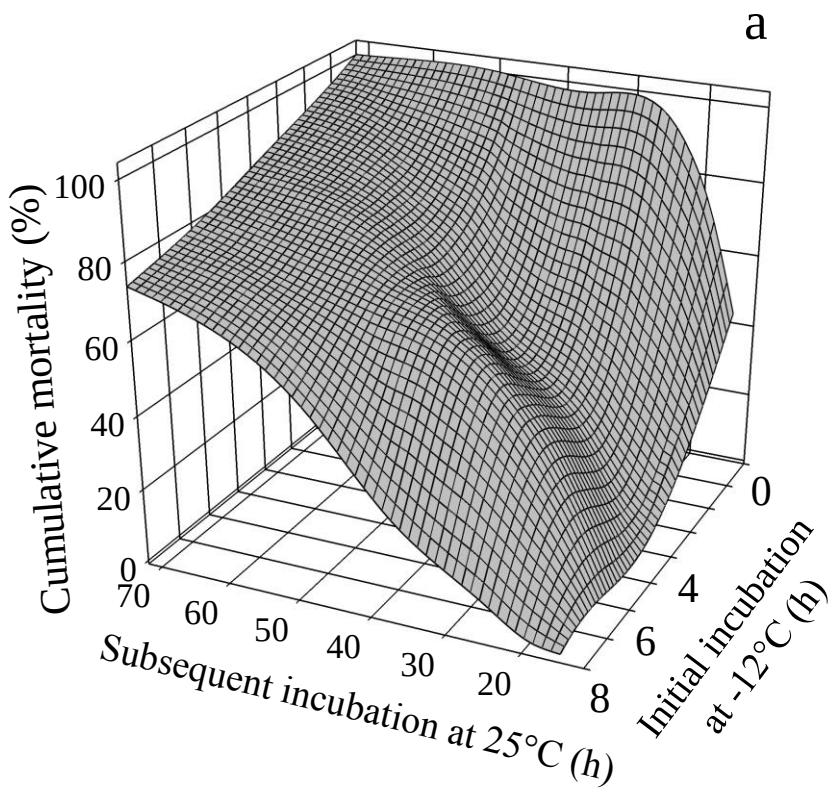
of positive control 15.9 (10.6−20.7) h and 26.2 (21.3−36.9) h, respectively (slope ± SE 0.12 ± 0.01) at 25ºC.

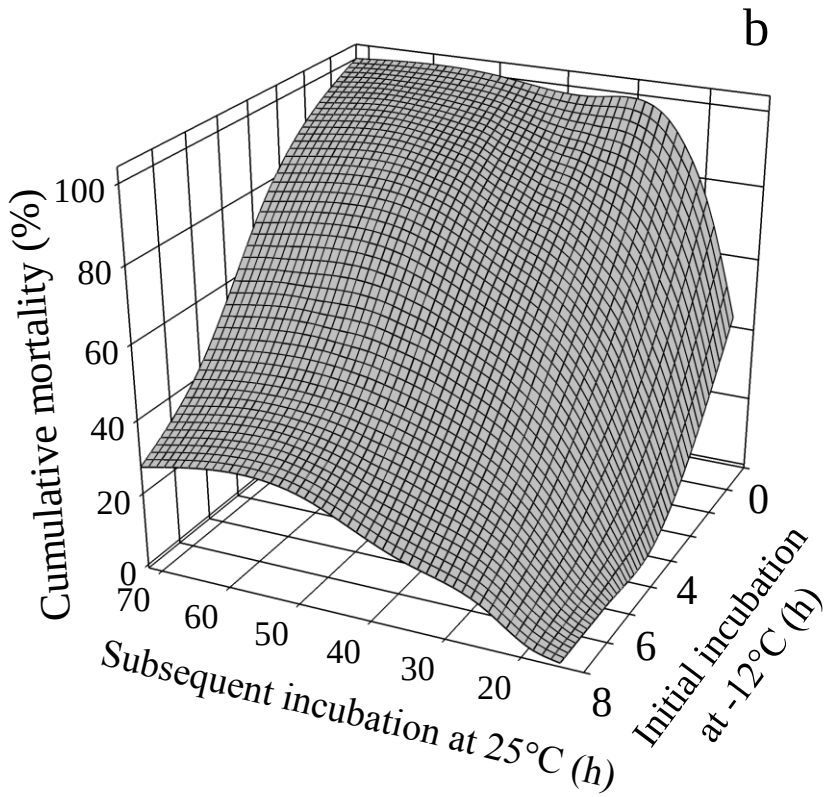
* suspended cysts defrosted in a water bath at 25ºC for 30 min; ** cysts defrosted overnight at 4ºC; *** values of mortality insufficient to calculate lethal times

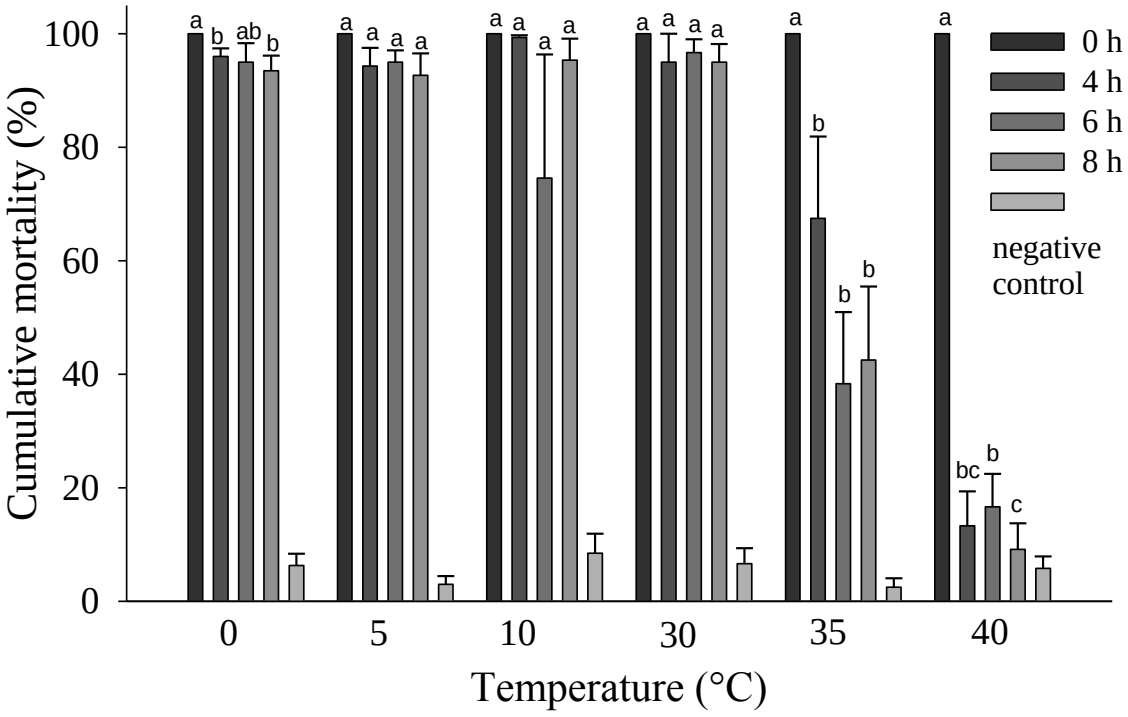
Table 2 - Relative mean number of dead *Aedes aegypti* third instar larvae (L3) ± standard error of the mean (SE) with oogonia of *Leptolegnia chapmanii* formed after exposure at different temperature (-12°C up to 40°C) and exposure time (4–8 h) and cumulative mortality of new L3 exposed to dead larvae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

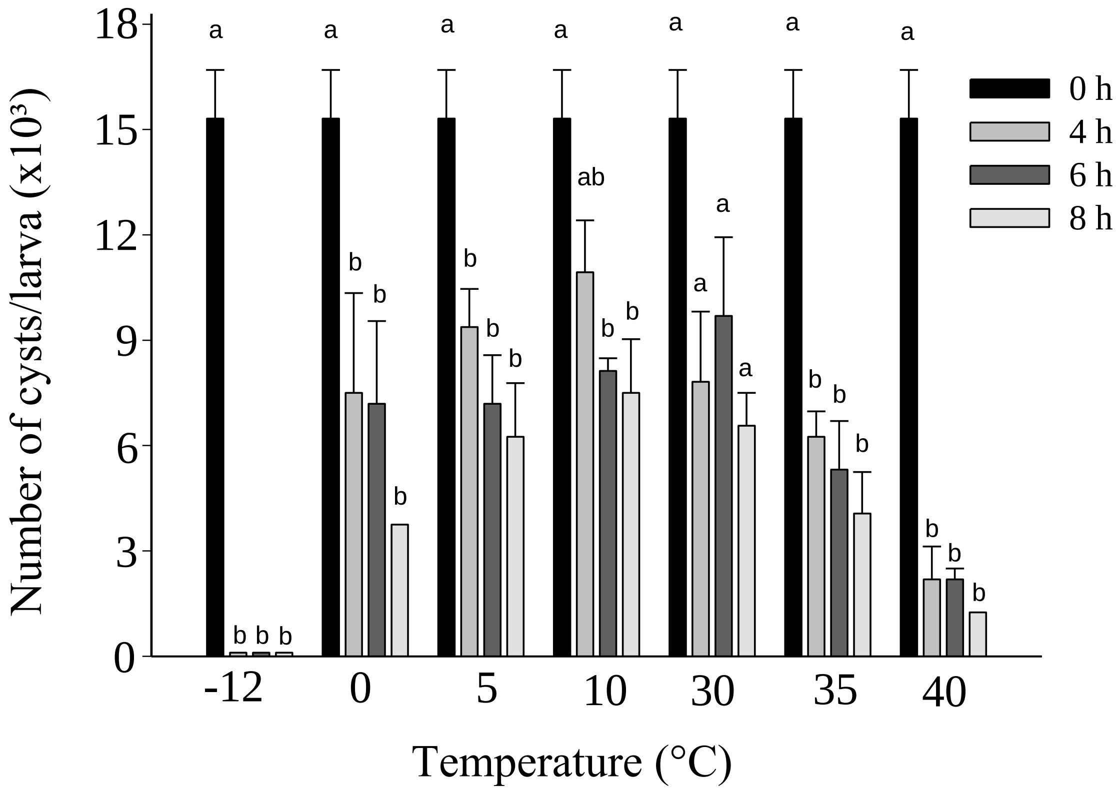
Temperature (°C)	Hours	Percentage of larvae with oogonia (± SE)	Mortality % (± SE) ****
-12**	4	25 ± 10.2	45 ± 15.5
	6	37.5 ± 16.1	41.3 ± 20.8
	8	0	19.4 ± 5.6
-12***	4	12.5 ± 14.4	70.6 ± 13.4
	6	62.5 ± 16.1	27.5 ± 9.8
	8	0	43.3 ± 16.9
0	4	62.5 ± 12.5	73 ± 23
	6	75 ± 10.2	90.6 ± 6.5
	8	68.8 ± 6.3	87.5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


a
Cumulative mortality (%)
100
80
60
40
20
0
70
60
50
40
30
20
8
Subsequent incubation at 25°C (h)
0
4
6
Initial incubation at -12°C (h)


b
Cumulative mortality (%)
100
80
60
40
20
0
70
60
50
40
30
20
8
Subsequent incubation at 25°C (h)
0
4
6
Initial incubation at -12°C (h)




Number of cysts/larva (x10³)
18
15
12
9
6
3
0
-12
0
5
10
30
35
40
Temperature (°C)
0 h
4 h
6 h
8 h
a
b
ab