

Infective capacity of the commercial nematode *Steinernema carpocapsae* parasitizing *Aedes aegypti* mosquito larvae in Yucatán México

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Summary

Dengue is currently the most important arbovirolosis in the world, affecting over 120 countries in both tropical and sub-tropical areas, with 2500 million people at risk. Due to global warming, it is expected that *Aedes aegypti* will be able to survive at higher latitudes, increasing the number of people at risk. Therefore, it is crucial to develop control strategies for the mosquito to prevent its environmental impact. The objective of this paper was to determine the concentration of entomopathogenic nematode (EPN) *Steinernema carpocapsae* that produces the highest percentage mortality of *Ae. aegypti* larvae under normal environmental conditions in Yucatán (6.9 – 8.7 mg/l; temperature 25.9 – 31.5°C; pH 7.20 – 8.10). The *S. carpocapsae* mode of action consists of *Xenorhabdus nematophila* bacterial release in the haemocoel of the insect, which produces septicaemia and consequently kills the mosquito larvae 24 – 48 h after intake. As for the methodology, a commercial EPN strain was used for experimental infection. The experimental design was a 70-h static bioassay that exposed 10 mosquito larvae per experimental unit (400-ml flask and five replicates per treatment) to four nematode concentrations (1250, 2500, 3125 and 3750 nematodes per ml) plus controls. The best treatment was with 1250 nematodes/ml, which caused 54 % mortality with respect to controls after 70 h (one-way ANOVA; $F_{5,72, 0.05}$; $p < 0.04$). We concluded that *S. carpocapsae* is a promising biological control tool for killing *Ae. aegypti* larvae at an experimental level. However, it is still necessary to determine its performance at higher volume scales in real-life conditions.

Keywords: *Aedes aegypti*; Biological control; Dengue; Entomopathogenic nematode; *Steinernema carpocapsae*

Introduction

Dengue is currently the most important arbovirolosis in the world, affecting over 120 countries in both tropical and sub-tropical areas, with 2500 million people at risk (Rizwan *et al.*, 2024). Due to global warming, it is expected that *Aedes aegypti*, the vector of dengue

fever in the Americas, will be able to survive at higher latitudes, increasing the number of people at risk (Childs *et al.*, 2024). In México, dengue fever has been present since the 1970s, with yearly variations in its incidence rate and epidemic outbreaks of different magnitudes, chiefly in states of the south and southeast regions, the Pacific and the Gulf of México (DeAntonio *et al.*, 2021).

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In 2023, the National Liaison Center for the National Health Regulations of México notified 31,549 dengue cases (https://www.gob.mx/cms/uploads/attachment_data/filename/878786/Pano/dengue/52/2023) and particularly the State of Yucatán are considered endemic zones with the highest incidence of dengue fever with 10,460 confirmed cases (<https://www.who.int/es/emergencies/disease-outbreak-news/item/2023-DON475>).

Currently there is a vaccine, but it is only aimed at people aged 9 – 45 years with previous history of dengue virus infection (Tully & Griffiths, 2021; Pintado & Fernandez, 2023). Given these limitations, the public health strategies are oriented to removal of water from transient aquatic habitats where female mosquitos lay eggs and larvae mature to adulthood (e.g., used tires, plastic containers, and crypt vases) and control the vector *Ae. aegypti* (Reyes-Castro *et al.*, 2017). Therefore, it is crucial to develop control strategies against the mosquito, avoiding the use of pesticides such as malathion and chlorpyrifos, which have an environmental impact and cause neurological disorders that are serious problems for domestic animals (dogs, cats, livestock) and vulnerable human groups (pregnant women and babies, children and older people) (Elmorsy *et al.*, 2022).

Biological control by using entomopathogenic nematodes is an alternative for fighting the virus of dengue fever because they are non-contaminating, as opposed to the toxic chemical insecticides that have been used in the past, which have polluted the environment and damaged the fauna, flora and human health (Abd-Elgawad, 2024). There are other agents of biological control that offer alternatives for combatting mosquitoes, such as entomopathogenic bacteria *Wolbachia* spp. in Australia (Hoffmann *et al.*, 2024), parasitic nematodes *Romanomermis iyengary* in Africa (Abagli *et al.*, 2019), larvae-eating fish *Gambusia puncticulata* and *Poecilia reticulata* in Cuba (Fimia & Osés, 2021) and entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* (Renuka *et al.*, 2023).

In laboratory tests, the entomopathogenic nematode *Steinernema carpocapsae* infects over 250 species of insects and may attack the developmental stages of larvae, pupa and adult insects (Elqdhay *et al.*, 2024). *S. carpocapsae* is produced industrially to kill leaf-dwelling pests from the families of Noctuidae (noctuids), larvae of Pyralidae (e.g. *Duponchella fovealis*), Tipulidae (crane flies), great pine weevil (*Hylobius abietis*), Coleoptera (beetles), Orthoptera (e.g. mole crickets) and larvae of *Capnodis tenebrionis* (<https://www.koppert.com/products/products-pests-diseases/capsanem/>).

The lifecycle of the species of the genus *Steinernema*, described by Glaser (1932), Boven (1937), Dutky (1974) and Poinar (1979), includes three infective juvenile (IJ) forms, all of them carrying symbiotic bacteria in their intestines. This symbiotic association confers both *S. carpocapsae* and its bacteria *Xenorhabdus nematophila* a double function as bioinsecticides (Cortés-Martínez *et al.*, 2023). When the nematode comes into contact with host in-

sect larvae, penetrates them by their natural orifices (mouth, anus, spiracles) and the body cavity releases the bacteria *X. nematophila* (Neira-Monsalve, 2020). The bacteria contain a toxin known as Txp40. This toxin has been shown to be active against a variety of insect species and grows rapidly, causing septicaemia and death of the larvae (Kinkar *et al.*, 2024).

Steinernema carpocapsae is a terrestrial nematode with an ambush feeding strategy and apparently low tolerance to high temperature (> 25°C) (Lortkipanidze *et al.*, 2016). Previous reports suggest that the infective larval stages of terrestrial entomopathogenic nematodes can infect mosquito larvae in aquatic environments, as in the case of *Steinernema abassi* infecting the larvae of *Ae. aegypti* with 97 % mortality (Dilipkumar *et al.*, 2019).

Thus, we hypothesized that *S. carpocapsae* could be a practical biological control agent of mosquito larvae because of its wide commercial availability and its symbiotic association with bacteria *Xenorhabdus nematophila*, which is highly pathogenic for pest insects.

Clearly, it is necessary to determine if these nematodes are capable of infecting mosquito larvae in at least a concentration to be used as a biological control tool in Yucatán and, by extension, in other tropical countries around the world. It is also true that it would not be expected to reach 100 % larvae but at least a certain level with which, together with pesticides or used within integrated pest management (IPM) to decrease the probability of transmission by infected mosquito females. Therefore, the objective of this paper was to determine, through an experimental approach, the infective capacity of the entomopathogenic nematodes *S. carpocapsae* for biological control of *Ae. aegypti* mosquito larvae under non-controlled environmental conditions of Yucatán, México.

Material and Methods

Mosquito larvae were obtained from *Ae. aegypti* eggs graciously donated by Dr. Hideyo Noguchi at the Centro de Investigaciones Regionales (Universidad Autónoma de Yucatán). The eggs were laid in small plastic jars with water, and the larvae that emerged were used in the experiment. Only larvae in the third and fourth instars were used, because these stages are more susceptible to infection by *S. carpocapsae* (Liu *et al.*, 2020). The experiment was undertaken using the normal environmental water conditions for Yucatán (see Table 1).

Nematodes were purchased from Koppert México, S.A. de C.V. (Sociedad Anónima de Capital Variable). Boxes containing 500 million larvae in the third stage were refrigerated at 4°C until the experiment began (no more than a week after acquisition). The nematodes were activated by adding tapwater to a Petri dish with a small piece (ca.1 cm²) of inert material containing the nematodes in a dormant state. The nematodes were divided into two groups in translucent 1-l plastic jars: 20 jars for the experimental treatment and 20 for the controls without nematodes. In the experimental group, the 20 treatment jars were randomly assigned one

Table 1. Cumulative mortality (%) of *Aedes aegypti* larvae due to the exposure to different concentrations (nematodes per ml) of infective third stage larvae of *Steinernema carpocapsae* and the values of the physicochemical parameters from the plastic containers mimicking crypt vases.

Time (h)	Cumulative mortality (%)	Oxygen (mg/L)	Temperature (°C)
Control without nematodes			
10	0	5.12	26.30
22	4	1.73	29.00
34	8	1.24	26.60
46	10	1.10	28.00
58	12	0.97	25.60
70	12	0.60	27.70
1250 nematodes/ml			
10	0	1.78	26.20
22	6	1.41	29.10
34	20	1.05	27.00
46	36	1.03	28.00
58	48	1.02	25.00
70	54	1.02	27.70
2500 nematodes/ml			
10	0	1.69	26.20
22	2	1.56	29.40
34	12	1.28	26.60
46	18	1.10	28.20
58	26	1.02	25.00
70	36	0.68	27.60
3125 nematodes/ml			
10	0	1.61	26.00
22	2	1.28	29.60
34	10	1.05	26.90
46	16	0.88	28.20
58	20	0.50	25.30
70	26	0.29	27.50
3750 nematodes/ml			
10	0	1.54	26.00
22	2	1.10	29.40
34	10	0.71	26.80
46	14	0.52	28.00
58	16	0.48	25.00
70	22	0.33	27.70

of four concentrations – 1250, 2500, 3125 or 3750 nematodes/ml (n/ml) – with five replicates per treatment. Ten larvae of *Ae. aegypti* were randomly assigned to each jar and filled with tapwater to 400 ml. Once the nematodes were inoculated, oxygen concentration (mg/l), temperature (°C) and mortality (%) were recorded every 2 h for 70 h. No environmental parameters were controlled and dead larvae (those not responding to the touch of a fine paintbrush [000-gauge]) were immediately removed with the paintbrush and placed in vials with 10 % buffered formalin for histology (several larvae per vial) or in 96 % ethanol for polymerase chain reaction (PCR; one larva per 1.5-ml vial).

For DNA extraction, each infected mosquito larva was cut in half using a sterile scalpel to reduce the extraction time according to the protocol of the DNeasy Blood and Tissue Kit (Qiagen™). The internal transcribed spacer (ITS) region of ribosomal DNA was amplified by PCR. All individual tubes were kept on ice. For the PCR mix in each tube we added the following: 5 µl of 10× PCR buffer (7.5 mM magnesium [pH 8.5], 0.5 µl of dNTP mixture [10 mM each], 0.5 µl of forward primer, 0.5 µl of reverse primer, 0.2 µl of GoTaq® DNA polymerase (5 µl/µl), 38.30 µl of distilled water and 5 µl of genomic DNA for a final volume of 50 µl. The primers used in this study were those reported by Hominick *et al.* (1997) and the expected product size was in the 900 – 1000 base pair (bp) range. The primer sequences were as follows: forward AB28 (5-ATATGCTTAAGTTCAGCGGT-3) and reverse TW81 (5-GTTTCCGTAGGTGAACCTGC-3), both amplified for the ITS region. All PCR reactions were run in an Axygen® MaxyGene™ II thermocycler under the following amplification conditions: one cycle of pre-denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 40°C for 30 seconds, annealing at 45°C for 60 seconds, extension at 72°C for 90 seconds and a final extension at 72°C for 5 minutes. The PCR products were checked by electrophoresis on a 1 % agarose gel using 1× TAE buffer at 85 V

for 45 min in a BioRad Sub-Cell®GT agarose gel electrophoresis system using a Promega® DNA ladder of 1 kb molecular weight as a reference. The PCR products were visualized in a BioDoc-It® Imager.

For histology, each *Ae. aegypti* larva was dehydrated four times in 100 % ethanol for 1 h, twice in chloroform (1 h each) and embedded in paraffin twice (1 h and then 2 h). Three sagittal sections 5-mm thick were cut from each specimen. Tissue slides were stained with haematoxylin and eosin, and Gram-Humberstone staining procedures were used to detect the nematodes (Soto-Rodríguez *et al.*, 2012). The sagittal sections were fixed to glass slides and mounted in Canada Balsam; then digital photographs were obtained with an Evolution MP colour digital camera (Media Cybernetics™) mounted on an Olympus™ BX50 optical microscope.

One-way ANOVA was used to determine whether at least one of the concentrations of nematodes produced a significant increase in mortality of the mosquito larvae. Unlike the temperature, oxygen concentration varied markedly throughout all treatments, including controls (Table 1), and was considered a variable that could affect nematode performance. However, the concentration of oxygen could not directly affect mosquito larvae mortality because they have siphons to breathe at the water surface. The assumption of normality of the mosquito larvae mortality data and the physico-chemical variables was verified using Wilk-Shapiro rankit plots (Sokal & Rohlf, 1995), obtaining values of 0.8 – 1 in all cases. Homoscedasticity was verified using Bartlett's test. Post hoc differences in mean values of mortality were determined using the LSD Fisher method at $p < 0.05$.

Ethical Approval and/or informed consent

The authors declare that they have complied with all applicable ethical standards.

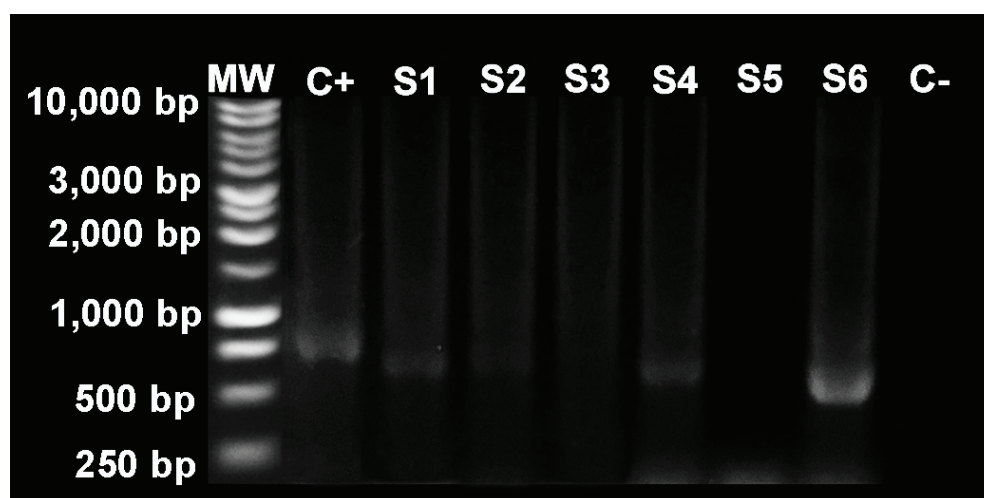


Fig. 1. PCR results of the experimental infection of *Aedes aegypti* larvae with the nematode *Steinernema carpocapsae*. PCR products were separated using 1% agarose gel electrophoresis. MW = molecular weight marker, 1,000 bp; C+ = positive control; C- = negative control; S1 to S6 included the DNA of individual *A. aegypti* larvae infected with *S. carpocapsae*.

Results

Polymerase chain reaction (PCR)

PCR detected genetic material of *S. carpocapsae* in *Ae. aegypti* larvae. In fact, 1,000 bp fragment was amplified at approximately equal intensity as the positive control and in the same range (900 – 1,000 bp) suggested by Hominick *et al.* (1997) as positive for *S. carpocapsae* (Fig. 1).

Histology

Nematodes were able to infect the mosquito larvae, as seen in Fig. 2, where infective juveniles (IJs) of *S. carpocapsae* appear in the mid-gut of the larva of *Ae. aegypti*. Melanized capsules were also visible in the gastric caeca region surrounding the nematodes (Fig. 2).

Statistical analysis

Table 1 shows the cumulative mortality (in percentage) of mosquito larvae for the four nematode concentrations and controls over time, as well as the physicochemical parameters obtained

during the experiment. The highest percent mortality produced by *S. carpocapsae* was 54 % at 70 hr post exposure with 1,250 nematodes per ml (n/ml) and 36 % at 70 hr post exposure with 2,500 nematodes per ml (Table 1). The cumulative mortality of mosquito larvae due to nematode larvae was significant when controlling for the effect of oxygen (one-way ANOVA; $F_{5,23} = 7.27$; $P = 0.002$). The mosquito larvae cumulative mortality was significant for 1,250 and 2,500 n/ml (LSD Fisher; $p < 0.05$) (Fig.3). However, mosquito cumulative mortality in the treatments between 3,125 and 3,750 n/ml were not significantly different from controls (LSD Fisher; $p > 0.05$) (Fig. 3). Temperature had no significant effect on the relationship between nematode concentration and percent mosquito larvae mortality (covariance analysis; $F_{5,23} = 0.04$; $P = 0.83$).

Discussion

The PCR and histology results indicated that *S. carpocapsae* was able to enter *Ae. aegypti* larvae (Figs. 1 and 2). Furthermore, the presence of this nematode in histological sections of the mosquito larvae body cavity suggested that it, along with its symbiotic

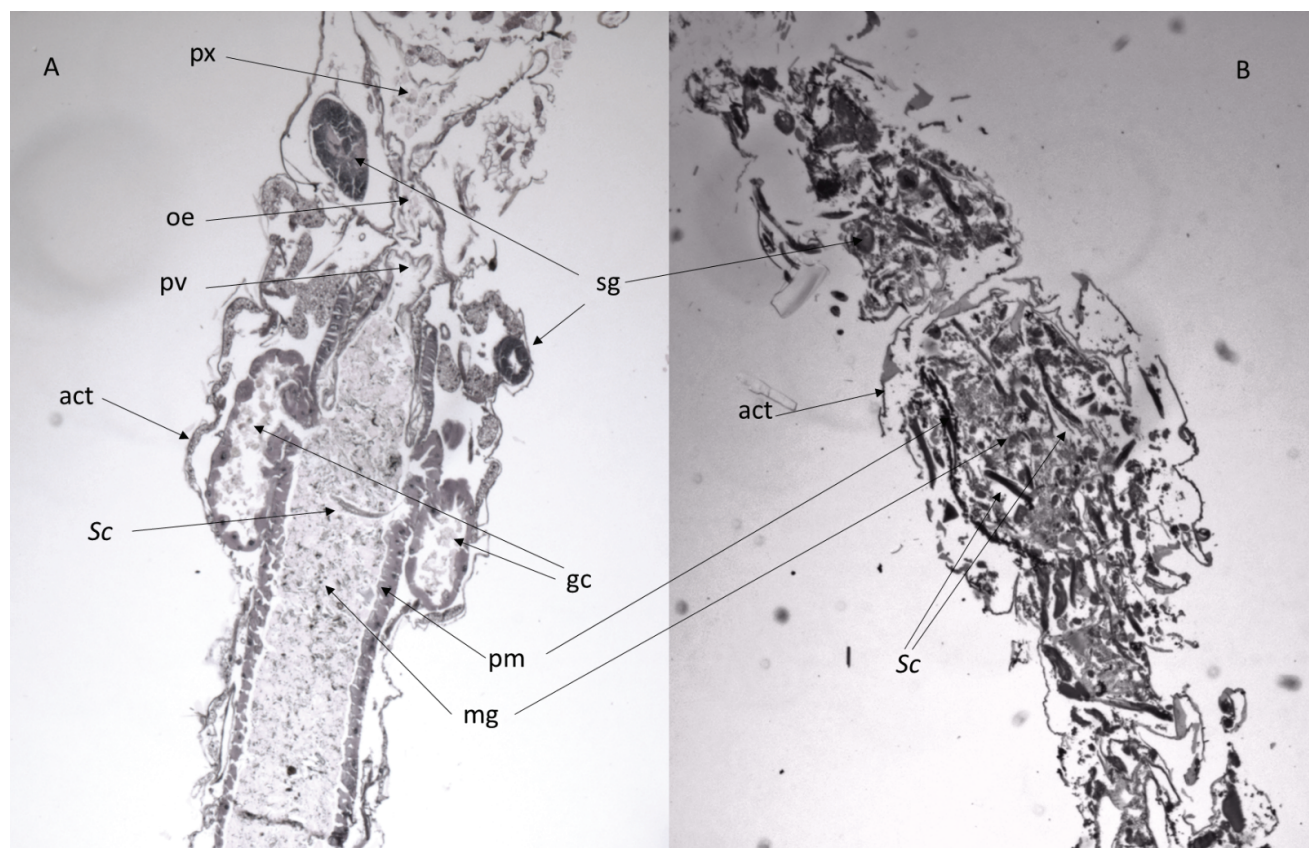


Fig. 2. Histological sections of *Aedes aegypti* mosquito larvae experimentally infected with the entomopathogenic nematode *Steinernema carpocapsae*. A) mosquito larva with light infection. Note the presence of the infective third stage of *S. carpocapsae* in the midgut (mg) of the larva. B) Heavily infected mosquito larvae with third stage larvae of *S. carpocapsae* in the midgut (mg). Note that the peritrophic membrane is necrosed and heavily infected by bacteria presumably *Xenorhabdus nematophila*. Lettering: act, annular chitinous thickening; px, pharynx; oe, oesophagus; pv, proventriculus; Sc, *Steinernema carpocapsae*; sg, salivary glands; gc, gastric caeca; pm, peritrophic membrane; mg, mid-gut. Scale bar: 100 μ m.

bacteria *X. nematophila*, was responsible for the septicaemia and mosquito larvae mortality. The significant increase of cumulative mortality of mosquito larvae (54 %) in jars containing 1250 and 2500 n/ml can be attributed primarily to the presence of the nematode and its symbiotic bacteria. However, mosquito larvae mortality was not significantly different from the controls in the largest nematode concentrations (3125 and 3750 n/ml).

In jars containing 1250 and 2500 n/ml (highest mortality levels), once the mosquito larvae fed, IJs showed a grade of destruction caused by the mouth movements in the larva proventriculus or mid-gut (Fig. 2). In all likelihood, melanized capsules were detected as a result of the defensive response of the mosquito larvae to low numbers of nematodes (Fig. 2). Mosquito larvae were not dissected for counting the amount of nematodes; instead, the study focused on the PCR and histology. However, it was assumed that because Welch and Bronskill (1962) also employed *S. carpocapsae* to infect *Ae. aegypti*, similar numbers of nematodes should be found in low and high levels of infection in the individual larvae that were exposed to 1250 and 2500 n/ml. Welch and Bronskill (1962) found that low-level infections (< 11 IJs) resulted in encapsulated *S. carpocapsae*, nematode removal by faeces or moulting

and mosquito larvae survival (Liu *et al.*, 2020). As the mortality rate was 54 %, it may well be possible that certain mosquito larvae presented a lower number of infections (Table 1). In contrast, infections caused by a high number of nematodes (presumably > 11 IJs per infected larva) allowed the release and growth of *X. nematophila* in the larvae body cavity and consequent septicaemia. Therefore, the most likely explanation for mosquito larvae mortality in jars containing 1250 and 2500 n/ml was that it was due to *S. carpocapsae* and its symbiotic bacteria in the first 48 h of infection (Table 1). Similar results infecting *Ae. aegypti* with *S. carpocapsae* were found by de Oliveira-Cardoso *et al.* (2015) and Dilipkumar *et al.* (2019).

The mortality rate (54 %) that was reached while exposing the *Ae. aegypti* larvae to 1250 n/ml was paradoxical because this nematode is well known for using an ambush feeding strategy with low tolerance to high temperatures (> 25°C) (Lortkipanidze *et al.*, 2016). Therefore, it may be possible that the mosquito larvae's behaviour could enhance infection. Kinney *et al.* (2014) have accurately described the *Ae. aegypti* larvae's behaviour based on three factors: wriggling; stationary filter feeding at the water surface; and substrate browsing. As expected in vases kept in crypts, sub-

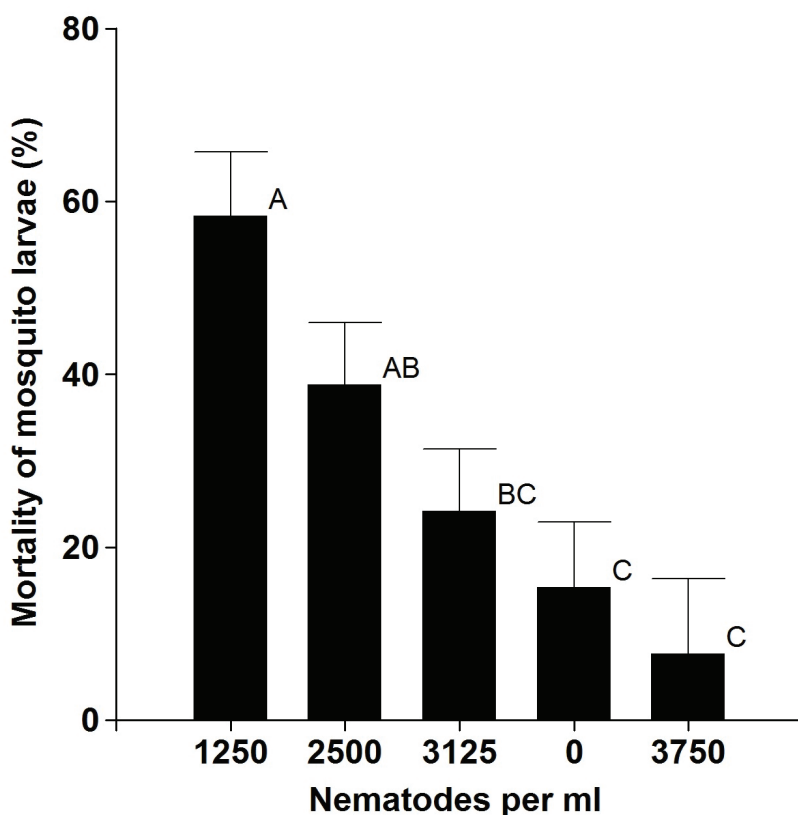


Fig. 3. Cumulative mortality (in percentage) of the larvae of *Aedes aegypti* exposed to different concentrations of the entomopathogenic nematode *Steinernema carpocapsae*. The black columns represent the mean values of the cumulative of mortality (%), and the error bars are the standard deviation (SD) values. Letters on the columns represent the posthoc difference in mean values of the percentage of mortality, which were determined using the LSD Fisher method at $p < 0.05$. Columns with a common letter were not significantly different ($p < 0.05$).

strate browsing actively increases after spotting the bottom of the container under food shortage. Therefore, it could be thought that such feeding behaviour increases the possibility of infection by the nematode. The aforementioned explanation agrees with Kunkel *et al.* (2006), who pointed out that the biology of the entomopathogenic nematode and its host should coincide to successfully cause an infection.

In the experimental jars, the water temperature (25 – 29.6°C) was not a limiting factor for *S. carpocapsae*. As Table I shows, all the experimental data were collected and kept in the shade. It is still necessary to determine if nematodes are able to survive and kill mosquito larvae when exposed to water temperatures of 26.1 – 29.6°C (79 – 85.3°F) and direct sunlight (<http://www.watertemperature.org/Yucatan-Basin-Geo.html>). The extant literature with regard to *S. carpocapsae* temperature is restricted to terrestrial environments with optimal infection at 25°C (e.g. Ali *et al.*, 2007). Nevertheless, the results of Ali *et al.* (2007) indicate that IJs and adults of *S. carpocapsae* present a high survival rate (76 %) at 25 – 30°C. The water containers employed during the current study were not warmed beyond this range (Table 1). In the same way, Devindrappa *et al.* (2018) showed that in a temperature range of 25 – 30°C, IJs of *S. carpocapsae* and *H. indica* are able to penetrate and reproduce in *Galleria mellonella* larvae, with *S. carpocapsae* being more tolerable to high temperatures. The nematode infection rates must be studied further, especially for vases kept in crypts in the shade, as the survival rate of *S. carpocapsae* at 35°C decreases when the temperature increases.

Although the nematode levels of 1250 and 2500 n/ml resulted in significant mosquito larvae mortality, these represent a very large number of nematodes per ml. In Latin American cemeteries, vases placed in crypts have volumes of 0.25 – 2 l (Vezzani, 2007). Therefore, based on concentrations of 1250 and 2500 n/ml, the amount per vase is calculated at approximately 125,000 to 1,000,000 nematodes. Commercial nematodes are sold in units of 500 million *S. carpocapsae* IJs per box, which means that a single box would have enough nematodes for 500 vases (assuming a 1-l volume). However, depending on the cemetery size, it may be possible to accommodate 2500 – 6000 vases (Vezzani, 2007). With the purpose of reaching the highest mortality rate in the field, it will also be necessary to determine the minimum number of required nematodes.

The highest concentrations of nematodes (3125 – 3750 n/ml) were not effective at producing a higher mosquito larvae mortality rate. There are two possible explanations for this: the ecological intraspecific interactions (e.g. O'Callaghan *et al.*, 2014) among the individual nematodes or the environmental conditions (temperature, oxygen, pH) were expressly uncontrolled. Even though the study did not measure the intraspecific competition among *S. carpocapsae* IJs, it is highly likely that low oxygen concentration resulted in a major impact on the nematodes' behaviour. At low oxygen levels, *S. carpocapsae* IJs become dormant and unable to infect mosquito larvae (Qiu & Bedding, 2000). The observed

mortality levels (54 % cumulative mortality 70 h post-exposure) were compared to those obtained by Ulvedal *et al.* (2017) through *Heterorhabditis bacteriophora* in field tests (> 50 % mortality on *Culex quinquefasciatus*). For this reason, the suggestion is to subject the nematodes to field exposure for accurate biological control of *Ae. aegypti* mosquito larvae, especially in shaded crypt vases located in cemeteries in Yucatán, as these places represent suitable habitats for mosquito larvae. It is important to point out that even when the vases have no flowers, these are filled with water daily during the rainy season, saturating with different species of mosquito larvae. The aforementioned situation can be confirmed in México City (Díaz-Badillo *et al.*, 2011), Campeche (Sosa-Cabrera, 2008), Argentina (Vezzani *et al.*, 2007), the United States (Dhillon & Mulla, 1980) and many other places all over the world (see Table 1 in Vezzani, 2007). The next step towards research could be a field assessment in local cemeteries using nematodes as biological control agents of *Ae. aegypti* mosquito larvae.

The current work demonstrates that *Ae. aegypti* larvae can be infected by *S. carpocapsae* under tropical weather conditions. It also suggests that the most viable explanation for the relatively high mortality rate (54 %) was the septicaemia produced by *X. nematophila*. However, it remains difficult to develop an alternative method that can connect live nematodes with mosquito larvae in the field. Therefore, future research should focus on the toxic compounds produced by *X. nematophila* or other local nematode/bacteria complexes that might kill mosquito larvae. Non-native agents that may act as a biological control (nematodes, bacteria or their metabolites) should be as host-specific as possible in order to lessen any potential damage to useful insects, such as local species of bees or other pollinators.

Conflict of interest

There is no conflict of interest in this work.

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