Larvicidal Activity of Entomopathogenic Nematodes Isolated from Mexico against *Aedes aegypti* (Diptera: Culicidae)[1]

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Abstract Entomopathogenic nematodes (EPNs) are widely used agents of biological control, mainly targeting soil-inhabiting insect pests. Reports indicate that these terrestrial EPNs are also able to infect the aquatic larvae of mosquitoes. We isolated EPN strains (*Heterorhabditis bacteriophora* Poinar and *Steinernema carpocapsae* [Weiser]) from local soils at Saltillo, Coahuila state, Mexico. EPNs from these strains were produced in the laboratory in yellow mealworm (*Tenebrio molitor* L.) larvae, and their pathogenicity as infective juveniles (IJs) was tested against larvae of the yellow fever mosquito *Aedes aegypti* (L.) Third- and fourth-instar mosquito larvae were exposed to four concentrations of IJs (25, 50, 100, and 200 IJ/larva) of five strains of local EPNs in laboratory assays. All strains of EPN caused lethal infections in larvae (3–100%); in particular, strain M5 of *S. carpocapsae* caused 100% mortality at the 200 IJ/larva concentration, with a median lethal concentration (LC_{50}) of 42 IJ/larva ($LC_{90} = 91$ IJ/larva). Strain M18 of *H. bacteriophora* caused 73% mortality at 200 IJ/larva, with an $LC_{50} = 72$ and $LC_{90} = 319$ IJ/larva. IJs were produced by all strains in mosquito larvae, with a range of 66–239 IJ/mosquito larva (inoculated at 100 IJ/larva) across strains, suggesting that horizontal transmission might occur in the field. This represents the first report of native EPN strains from Mexico exhibiting pathogenicity against mosquito larvae. Native EPN strains should be further evaluated as potential biological control agents in mosquito management.

Key Words dengue, Zika, chikungunya, insect, biocontrol

The yellow fever mosquito *Aedes aegypti* (L.) (Diptera: Culicidae) is a vector of viruses causing human diseases. *Aedes* species can transmit chikungunya, dengue, yellow fever, and Zika viruses (Jansen et al. 2018; Martinez el al. 2019). It is estimated that each year 50–100 million infections of these diseases occur, and 2.5 billion people live in risk areas where transmission of these viruses is high (Vontas et al. 2012). Mexico is among the countries with the highest epidemiological risk (Peltonen et al. 2018).

Controlling mosquito populations is the best strategy to avoid the spread of these diseases (Wilk-da-Silva et al. 2018). The uncontrolled and indiscriminate use of chemical pesticides, however, leads to resistance, causing control program failures

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Table 1. Entomopathogenic nematode strains isolated from agricultural soils at UAAAN, Saltillo, Mexico, 2017.

Crop	Number (%) of Positive Soil Samples	Nematode Species	Strain(s)
Oats	0		
Pecan	1 (7)	*Heterorhabditis bacteriophora*	MZ6, MZ9
Corn (maize)	2 (14)	*Heterorhabditis bacteriophora*	M18
		Steinernema carpocapsae	M5
Weedy area around fields	1 (7)	*Steinernema carpocapsae*	B7

and an increased risk of disease transmission. Chemicals can cause environmental damage and impact nontarget organisms and human health (Bharati and Saha 2018). Therefore, it is necessary to generate efficient alternatives for the control of insect vectors through the use of biological control agents and natural products from plants and microorganisms (El-Akhal et al. 2014).

A possible alternative for the control of mosquitoes is the use of entomopathogenic nematodes (EPNs) of the families Steinernematidae and Heterorhabditidae, which are widely used as biopesticides for the control of insect pests (Shapiro-Ilan et al. 2016). EPNs can kill their hosts in less than 48 h due to their symbiotic association with Enterobacteria of the genera *Photorhabdus* and *Xenorhabdus* that are transported in the digestive tract of infective juveniles (IJs) (Campos-Herrera et al. 2012). The possibility of the use of EPNs for the control of mosquito larvae was documented first by Welch (1962), who indicated that *Steinernema carpocapsae* (Weiser) can infect larvae of *Aedes* spp. Different species of EPNs have additionally been reported to control mosquito larvae (Cardoso et al. 2016). The present study focused on determining the larvicidal activity of local Mexican strains of EPN against larvae of *A. aegypti* and evaluating progeny (IJs) production in mosquito larvae under controlled conditions.

Materials and Methods

The study was conducted in May 2018 in the Biological Control Laboratory, Universidad Autónoma Agraria Antonio Narro (UAAAN), Saltillo, state of Coahuila, Mexico. Strains of EPNs assessed belong to *Heterorhabditis* (MZ6, MZ9, and M18) and *Steinernema* (B7 and M5) that were originally obtained from agricultural soils at UAAAN on 15 May 2017 (Table 1) by using the insect bait technique (Bedding and Akhurst 1975; Sanchez-Pena et al. 2011). Fourth- and fifth-instar yellow mealworm, *Tenebrio molitor* L. larvae served as bait in soil samples (see Table 1) at room temperature (20–25°C). Soil samples with larvae were inspected every 2–3 d until dead larvae were observed with the characteristic appearance of EPN infection (e.g., wine-red coloration for *Heterorhabditis* and dark brown for *Steinernema*)

(Wang et al. 2014). Mealworm cadavers were collected, carefully washed in running tap water for 1 min, rinsed (if intact) with 70% ethanol for 20 s, followed with rinsing in distilled water, and transferred to modified White traps (Kaya and Stock 1997) until complete IJ emergence. Each strain was isolated from each infected insect by inoculating 500–1,000 IJs from these soil-exposed larvae onto 5 to 10 *T. molitor* larvae (4th and 5th instar) in plastic Petri dishes (100 × 15 mm) lined with moistened filter paper and maintained at 22–26°C. After IJ emergence (2–3 weeks), each strain was serially inoculated onto 10 *T. molitor* larvae at 200 IJ/larva in Petri dishes. After 48 h, insect cadavers from these inoculation dishes were also transferred to White traps (Kaya and Stock 1997) until progeny (IJ) emergence; IJs were maintained in tissue culture bottles at 20–25°C for a maximum of 10 d before use in bioassays (Stock and Goodrich-Blair 2012).

Nematode identification. The nematodes were identified to genus based on morphological characteristics and appearance of infected larvae (Poinar 1979, Kaya and Stock 1997). For DNA extraction, the methods described in Tanha Maafi et al. (2003) were followed. DNA was extracted from several dozen nematodes from each genus. For amplification of the internal transcribed spacer (ITS)-rRNA gene, the forward TW81 (5′-GTT TCC GTA GGT GAA CCT GC-3′) and reverse AB28 (5′-ATA TGC TTA AGT TCA GCG GGT-3′) primers were used. For the D2-D3 region of 28S rRNA gene, the forward D2A (5′-ACA AGT ACC GTG AGG GAA AGT TG-3′) and reverse D3B (5′-TCG GAA GGA ACC AGC TAC TA-3′) primers were used. For *Steinernema*, the D2-D3 region of the 28S rRNA gene was sequenced only. Voucher specimens were deposited at the Plant Pest Diagnostics Center, California Department of Food and Agriculture, Sacramento, CA.

Bioassay of insecticidal activity of EPN. Mosquito larvae used in the bioassays were from a laboratory colony of *A. aegypti,* originally collected from artificial rainwater deposits near the eastern limits of Coahuila state, Mexico. Specimens were identified using taxonomic keys (Ibáñez-Bernal and Martinez 1994). Dry fish food (Tetra®; Spectrum Brands Pet, LLC, Blacksburg, VA) was provided to larvae maintained at 27°C, 70% relative humidity, and diffuse natural light (Armbruster et al. 2000).

Each experimental unit (replicate) of the bioassay was a plastic container (5.5-cm interior diameter × 4.0 cm tall, total volume of 130 cm^3) (Envases Cuellar, Saltillo, Mexico) with 100 ml (final volume) of purified bottled water. Ten third- and fourth-instar *A. aegypti* larvae were added to each container. Treatments of 25, 50, 100, and 200 IJ/larva were added to the appropriate containers and maintained at 25°C. Even though numbers of IJs applied to groups of larvae were determined, we use the term "concentration" and not "dose" for treatments because the actual number of IJs invading individual mosquito larvae was not known. Each treatment (concentrations of each nematode strain) was replicated 6 times. Controls consisted of containers with mosquito larvae without EPNs in the water. After 72 h, dead larvae (e.g., those that did not show movement for 30 s after being touched with a fine painting brush) were counted. Additionally, dead larvae were photographed and recorded by video using a microscope equipped with a TrueDepth 12MP wide-angle camera (Apple Inc., Cupertino, CA).

Production of IJs by different strains of EPNs in *A. aegypti* larvae. The IJ development in mosquito larvae was observed for each strain, inoculated at 100 IJ/larvae. Six dead larvae were randomly selected from each nematode strain

treatment and transferred individually to a Petri dish (5-cm diameter) containing 3 ml of sterile distilled water and maintained at 25°C in total darkness. Once the IJs emerged after 8–10 d, 10 aliquots of 5 μl each were observed microscopically (10×), and the number of IJ/larva produced was determined. The production of new IJs implies that the IJs applied against larvae invaded them successfully, developed to the adult stage, and then reproduced, releasing a new generation of IJs.

Data analysis. Differences in larval mortality among treatment means were determined by multiple comparisons and adjustment using the Tukey-Kramer test. The concentration-mortality response regression was analyzed using log-probit analysis (Finney 1971). For each of the concentrations and strains, probit analysis was used to estimate the median lethal concentration (LC_{50}), LC_{90}, 95% confidence intervals, and chi-square values. Lethal concentration values with overlapping confidence intervals were not considered significantly different. The production of IJs was determined by analysis of variance followed by Bonferroni's test for mean separation. All analyses were performed with StatPlus version 7.1.1.0 (2020).

Results

Nematode identification. EPNs used in this study, originally baited from soil using *T. molitor* larvae, were identified by morphology as *Steinernema* and *Heterorhabditis* (Table 1). In particular, several *T. molitor* larvae infected by *Heterorhabditis* showed the typical wine-red coloration induced in terrestrial insects after lethal invasion by species of *Heterorhabditis* and their associated *Photorhabdus* bacteria (Kaya and Stock 1997). Sequences of the ITS-rRNA and D2-D3 of 28S rRNA genomic regions of the *Heterorhabditis* strains showed 100% identity to many isolates of *Heterorhabditis bacteriophora* Poinar from Mexico (e.g., MK421440.1), Argentina, the Middle East, and India (i.e., NCBI 2019) (Machado et al. 2019), reflecting the near-worldwide distribution of this species (Campos-Herrera et al. 2012). Obtained sequences *of H. bacteriophora* are registered in GenBank: the 28S D2-D3 rRNA region as accession MN191509 and the ITS-rRNA region as MN191510.

The D2-D3 region of 28S rRNA gene sequence of the *Steinernema* isolates matched with 100% identity that of isolates of *S. carpocapsae* from different areas of the world (e.g., Colombia, Iran, Switzerland, and Mexico) (Noujeim et al. 2011). It also matched the classical isolate of this species, DD-136 (Welch and Bronskill 1962). The 28S D2-D3 rRNA region sequence of *S. carpocapsae* obtained in this work is deposited in GenBank (accession MN503268).

Bioassay of insecticidal activity of EPN. The EPN strains tested successfully infected *A. aegypti* larvae and also reproduced in these hosts (Fig. 1) to various degrees. A video recording of *S. carpocapsae* strain M5 activity in infected *A. aegypti* larvae may be viewed online at https://www.youtube.com/watch?v=3y1t7HYJ49U&feature=youtu.be (Treviño-Cueto and Sanchez-Peña 2018).

The observed larvae presented typical symptoms caused by EPN infection (Shapiro-Ilan et al. 2016); particularly, infection by *H. bacteriophora* also induced the wine-red coloration in several mosquito larvae, as described (Kaya and Stock 1997). Adults and juveniles of both *S. carpocapsae* and *H. bacteriophora* could be observed in the larvae infected by each nematode (Fig. 1), as observed by Treviño-

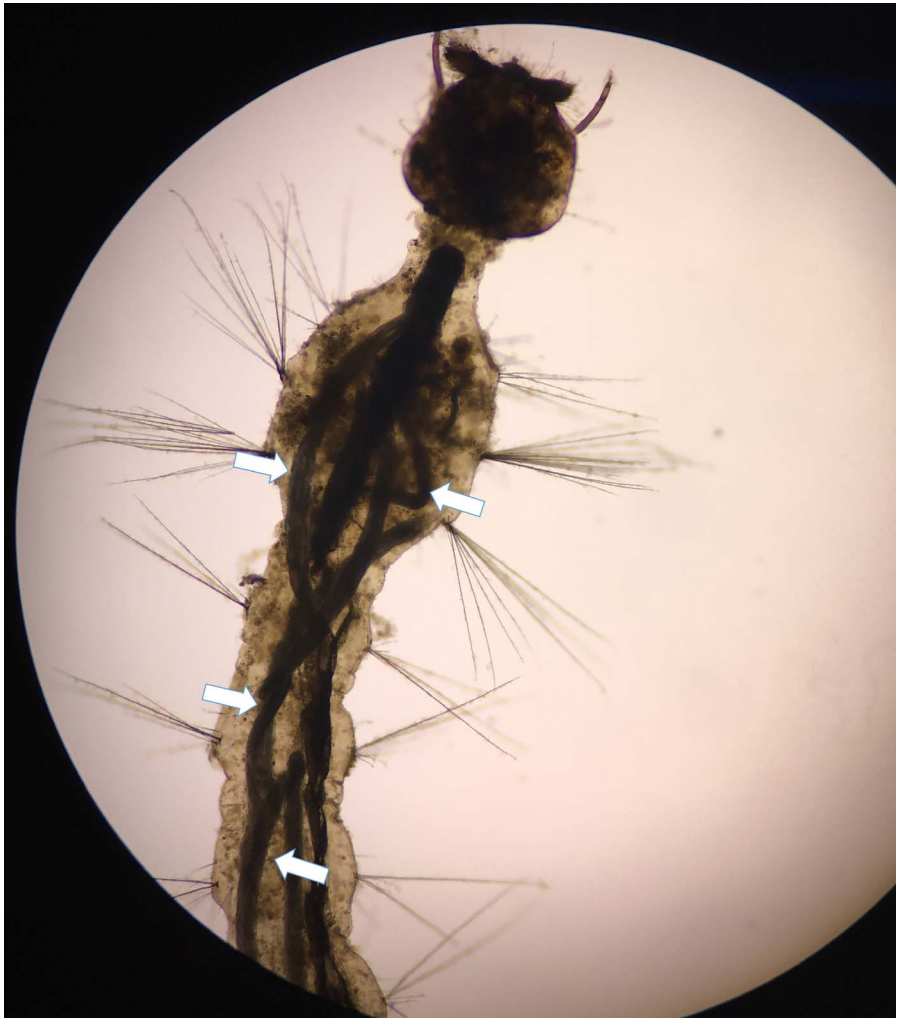

Fig. 1. Fourth-instar *A. aegypti* larvae infected by *S. carpocapsae* strain M5. Nematodes can be observed in the hemocoel of the insect (arrows).

Cueto and Sanchez-Pena (2018). There was no mortality of mosquito larvae in the control groups. Regarding concentration-mortality responses (Tables 2 and 3), mortalities ranging from 3.3 to 100% were observed in *A. aegypti* larvae after 72 h of exposure to different concentrations (IJ/larva) of *H. bacteriophora* (MZ6, MZ9, and MZ18) and *S. carpocapsae* (B7 and M5) (Table 2). *Steinernema carpocapsae* M5 induced the highest mortality (100%) at 100 and 200 IJ/larva; *H. bacteriophora* M18 caused 70% mortality at 100 IJ/larva and 73.3% at 200 IJ/larva ($F = 51.73$; df $= 1$, 23; $P = 0.05$).

Table 2. Mean percent mortality of *A. aegypti* larvae (3rd and 4th stage) 72 h after exposure to infective juveniles of native strains of entomopathogenic nematodes*.

Concentration (IJ/Larva)	Mean % Mortality (SD) of Strain:				
	MZ6	B7	MZ9	M18	M5
25	6.7 (5.2) i	6.6 (5.2) i	3.3 (5.2) i	8.3 (4.1) i	13.3 (8.2) i
50	20.0 (8.9) fghi	16.6 (8.2) fghi	16.6 (15.1) fghi	43.3 (10.3) de	53.3 (10.3) cd
100	36.7 (16.3) def	15.0 (5.5) fghi	25.0 (5.5) efgh	70.0 (26.8) c	100.0 (0) ab
200	45.0 (13.8) de	31.6 (9.8) defg	31.6 (7.5) defg	73.3 (12.1) bc	100.0 (0.0) a

* IJ, infective juvenile; B7 and M5, *S. carpocapsae*; the remaining strains, *H. bacteriophora*. Ten larvae were kept in 100 ml of water/replicate. Within rows and columns, means followed by the same letter are not significantly different (Tukey-Kramer test, $P = 0.05$).

Table 3. Lethal concentrations and confidence intervals 72 h after exposure of 3rd and 4th larval stage *A. aegypti* to infective juveniles of entomopathogenic nematodes *H. bacteriophora* and *S. carpocapsae.**

Nematode Strain	LC_{50} (IJ/larva) (LCIL–UCIL)	LC_{90} (IJ/larva) (LCIL–UCIL)	Slope (SE)	Intercept
S. carpocapsae (B7)	784.9 (259.3–5,681.9)	18,960.0 (2,034.9–533,236.9)	0.93 (0.22)	2.31
H. bacteriophora (MZ9)	422.6 (251.6–1,115.1)	4,948.9 (1,639.1–51,199.8)	1.20 (0.22)	1.85
H. bacteriophora (MZ6)	215.4 (159.0–351.3)	1,887.7 (895.6–7,296.2)	1.36 (0.20)	1.83
H. bacteriophora (M18)	72.0 (32.25–160.6)	319.7 (56.1–1,821.8)	1.98 (0.47)	1.32
S. carpocapsae (M5)	42.43 (38.06–47.0)	91.0 (79.6–107.7)	3.90 (0.32)	−1.34

* IJ, infective juvenile; LCIL, 95% lower confidence interval limit; UCIL, 95% upper confidence interval limit; SE, standard error of the slope. Ten larvae were kept in 100 ml of water/replicate.

Table 4. Mean production of infective juveniles in *A. aegypti* larvae by strains of entomopathogenic nematodes*.

Strain	Mean (SD) IJ Production/Mosquito Larva (SD)
H. bacteriophora M18	239.2 (7.4) a
S. carpocapsae M5	144.3 (7.5) b
H. bacteriophora MZ6	126.8 (18.5) bc
H. bacteriophora MZ9	114.3 (14.4) c
S. carpocapsae B7	66.5 (10.7) d
Control	0 e

* IJ, infective juvenile; SD, standard deviation. Means followed by different lowercase letters are significantly different (Bonferroni test, $P = 0.003$).

The results of the log-probit analysis are shown in Table 3. Most strains showed low slope values, with the exception of *S. carpocapsae* M5 ($\beta > 3.0$). However, there were significant differences among strains, as the 95% confidence intervals did not overlap among strains in several cases (Table 3). In general, strains M5 and M18 (*S. carpocapsae* and *H. bacteriophora*, respectively) had lower LC_{50} and LC_{90} and confidence intervals that were significantly different from those of the other strains. Among the strains tested, the highest and lowest LC_{50} values varied by a factor close to 20, and the highest and lowest LC_{90} values varied by a factor close to 200. The *P* values of the log-probit analysis indicated that most mortality lines fitted incompletely the adjusted linear log concentration-probit model ($P \geq 0.05$). Only the *S. carpocapsae* strain M18 closely fit the model ($P = 0.086$).

Production of IJs of EPNs in *A. aegypti* larvae. The reproduction and subsequent development of IJs of all of the tested EPN strains occurred in *A. aegypti* larvae (Table 4). Significantly greater numbers of IJs were produced by the *H. bacteriophora* M18 strain ($F = 37.45$; df = 1,4; $P = 0.05$). Numbers of IJs produced in mosquito larvae varied by a factor of 4× between strains producing the highest (M18) and lowest (B7) numbers. Strains M5 of *S. carpocapsae* and M18 of *H. bacteriophora*, which presented the first and second lowest LC_{50} and LC_{90} values, respectively, among strains (Table 3) also produced the highest numbers of IJs (Table 4).

Discussion

The results reported here demonstrate the potential role of the different strains of locally isolated EPNs as *A. aegypti* larvicides and indicate that mosquito-pathogenic strains of EPNs can be ubiquitous. The observed differences observed on virulence and reproductive success among the strains tested here. Researchers selecting EPN strains for eventual development as biopesticides should consider the variability detected here among the tested strains.

There is great interest in the development of new larvicides of biological origin that are capable of effectively controlling mosquito larvae without generating

insecticide resistance and that are harmless to the environment and human health. Infections of culicid larvae by rhabditid generalist EPNs were initially reported by Welch (1962), Welch and Bronskill (1962), Dadd (1971), and subsequently Poinar and Kaul (1982) under laboratory conditions and in field tests.

Among the many factors that might influence the pathogenicity of these rhabditid EPNs to mosquito larvae, their survival in the water environment is crucial; EPNs are terrestrial organisms, although they require a water-saturated microenvironment to invade insects (Glazer and Salame 2000). These authors reported a reduction in the viability of IJs of *S. carpocapsae* after 36 d at 25°C, whereas Andaló et al. (2010) reported high survival levels (>85%) after 40 d in water at 16°C for *S. carpocapsae* and *Heterorhabditis* sp. Therefore, we did not consider relevant the periods (<10 d at 20–25°C) of IJ storage in water before tests in the present work.

Invasion of mosquito larvae by IJs is assumed to occur through the mouth. After application to water, these nematodes sink to the bottom of the container and do not swim but instead crawl on the bottom of the water deposit (Treviño-Cueto and Sanchez-Pena 2018); therefore, invasion of mosquito larvae appears to occur after ingestion of IJs by these substrate feeders. Also, the spiracles and anus of mosquito larvae (additional portals of invasion for EPN in terrestrial insects) are in a raised position and rarely touch the substrate, where EPN would be some minutes after application. Therefore, invasion through the anus or spiracles appears less likely in these insects. We observed numerous IJs in the head capsule of invaded *Aedes* larvae, possibly indicating invasion through the mouth (Treviño-Cueto and Sanchez-Pena 2018).

Recently, in a similar fashion to that found in the present work, several reports have described both the lethal action of these and similar EPN species (Steinernematidae and Heterorhabditidae) against mosquitos and, in some cases, the reproduction of these EPNs in killed larvae. Cagnolo and Almiron (2010) reported the larvicidal effectiveness of *S. rarum* (Doucet) against *A. aegypti* larvae, with a mortality of 75% at a dose of 400 IJ/larva. Peschiutta et al. (2014) reported *A. aegypti* larval mortality of up to 84% with a dose of 750 IJ/larva of an Argentine strain of *H. bacteriophora,* which also produced progeny in killed insects (up to 168 IJ/larva). Also, in *A. aegypti*, Cardoso et al. (2016) showed that strain LPP1 (*Heterorhabditis indica* Poinar, Karunakar & David) caused mortality of up to 95%, with a mean production of 245 IJ/larva. The amounts of IJ production in *A. aegypti* larvae reported in the previous studies are within the range of our observations (Table 4). Chaudhary et al. (2017) found that *Steinernema kraussei* (Steiner) and *H. bacteriophora* were effective in inducing mortality of *A. aegypti* larvae with optimum activity between 20°C–30°C. They also reported a mortality rate of 100% by both EPNs in canal, tap, and sewage water. Aiswarya et al. (2019) reported that *Steinernema abassi* Elawad, Ahmad & Reid induced 97.3% mortality in *A. aegypti* larvae, 62% in *Anopheles stephensi* Liston larvae, and 76% in *Culex quinquefasciatus* Say larvae. Capinera et al. (1988) observed a positive correlation between the numbers of IJs inoculated in insect hosts and the production of progeny in hosts. In this work, there was an apparent positive relationship between strain virulence and IJ production; to our knowledge, this aspect has not been analyzed with mosquito larvae as hosts.

Additional research is needed to better evaluate the potential of EPNs, such as *S. carpocapsae* M5 and *H. bacteriophora* M18, as possible mosquito larvicidal

agents to be used in mosquito management. These organisms possess desirable traits, such as low production cost and negligible environmental impact. In addition, the strains tested here produced IJs in *A. aegypti* larvae, so horizontal transmission to new hosts and reproduction in mosquito habitats appears possible. This is the first report of native EPN strains from Mexico exhibiting pathogenicity against mosquito larvae; these EPN strains have potential as biological control tools within programs of integrated pest management of vectors in the country. Further research is required on the host-parasite interactions of EPNs and mosquito larvae, both in the laboratory and in the field.

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