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Indiscriminate ingestion of entomopathogenic nematodes and their symbiotic bacteria by *Aedes aegypti* larvae: a novel strategy to control the vector of Chikungunya, dengue and yellow fever

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Abstract: Third and fourth instar larvae of *Aedes aegypti* actively ingested entomopathogenic nematodes (EPNs) and their symbiotic bacteria, resulting in larval mortality. All six EPN species evaluated in this study were pathogenic to *Ae. aegypti* but varied significantly in their virulence. *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* were most virulent, *H. megidis* and *S. kraussei* showed the least virulence, whereas *H. downesi and S. feltiae* had intermediate virulence. Larval mortality was dose dependent for all EPN species. When using a dose of 100 infective juveniles (IJs) per larva, *H. bacteriophora* and *S. carpocapsae* caused 90%–100% mortality, whereas *H. downesi* and *S. feltiae* caused only 40%–60% mortality. Even when using 200 IJs/larva, *H. megidis* and *S. kraussei* caused a maximum of 30%–40% mortality. Some of the invasive EPNs were melanized, suggesting a strong humoral defense response by the *Aedes* larvae. The degree of melanization was quite variable; some EPNs were totally enveloped in a melanin sheath while others were partially coated with melanin. Melanization did not stop the EPN from multiplying and killing the *Aedes* larvae. IJs released from infected larvae would have the potential to infect healthy mosquito larvae. Also, both bacterial supernatant and bacterial cell suspension of *Xenorhabdus nematophila* caused >91% larval mortality after 48 h, whereas only the bacterial cell suspension of *Photorhabdus laumondii* was effective against the mosquito larvae. These data provides useful information on the potential use of EPNs and/or formulated bacterial cell suspensions in the control of the important urban and container-breeding mosquito, *Ae. aegypti,* and are a starting point for future simulated and actual field studies.

Key words: Entomopathogenic nematode (EPN) ingestion, *Aedes aegypti*, symbiotic bacteria, *H. bacteriophora*, *S. carpocapsae*

1. Introduction

Aedes aegypti, a mosquito species that breeds in many natural and artificial aquatic sites in urban situations, is a major vector of several human diseases such as dengue fever, Chikungunya, and yellow fever (Rodriguez et al., 2007; WHO 2009). Although yellow fever has been reasonably brought under control through its vaccine (Monath, 2005), no vaccine is available against dengue (Guzman, 2005; MacKenzie et al., 2004), which causes around 100 million infections (a 4-fold increase since 1990), 0.5 million cases of dengue hemorrhagic fever globally affecting 195 countries, and at least 40,000 deaths annually (Gubler, 2002; Zheng et al., 2021). Several factors such as unprecedented population growth (primarily in urban centres of tropical countries), the increased movement of viruses in infected humans through modern transportation and the lack of effective mosquito control are causing an alarming increase in the spread of the disease (MacKenzie et al., 2004). For example, before 1970, hemorrhagic fever was reported from only nine countries, whereas this number rose to 60 in 1999 (Kay and Nam, 2005). Similarly, the World Health Organization estimated 100–400 million infections annually with about half of world's population are at risk to dengue transmission risk, (WHO 2009, 2021). This problem could increase through adaptation of the mosquito to polluted water (Silva et al., 1999), which could make control a big challenge in the near future. Indeed, an outbreak of Chikungunya in the Indian Ocean islands is a reminder that even a relatively rare vector-borne disease poses a real and immediate threat to the health of millions of people (Higgs, 2006).

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Since an effective and commercially available dengue vaccine is not available and the immunology of dengue hemorrhagic fever makes vaccine development extremely challenging, dengue control has been limited to combating its vector (Guzman, 2005; MacKenzie et al., 2004). Vector control strategies include eradication of mosquito breeding sites by improving water storage and proper solid waste disposal, production of transgenic insects such as the self-limiting OX513A *Ae. aegypti* adult males (Patel et al., 2017) as well as treating breeding sites with chemical insecticides (Chadee, 2004; Gubler and Clark, 1994). Similarly, chemical insecticides are also being used against adult mosquitoes to suppress exceptionally high vector populations and/or dengue outbreaks (Reiter and Nathan, 2001; Rodriguez et al., 2007). Despite using the above strategies, complete eradication of breeding sites in urban and poorly developed areas is almost impossible. Moreover, continuous use of chemical insecticides has resulted in development of resistance to these insecticides in many countries (Rodriguez et al., 2007 and references therein). These factors, together with the concerns of risks to human health and increased environmental pollution caused by the synthetic insecticides, have prompted the search for safer alternative control strategies.

Microbial biological agents such as the bacteria, *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) and *Lysinibacillus sphaericus,* and the fungus, *Metarhizium anisopliae,* have been shown to be pathogenic to *Ae. aegypti* larvae and adults, respectively (Scholte et al., 2007; Alkhaibari et al., 2016). Similarly, parasitic mermithid nematodes are very effective against mosquitoes, but their high specificity towards their host and difficulties in mass production limit their potential for commercial use (Walgate, 1994). Earlier studies have demonstrated the efficacy of entomopathogenic nematodes (EPNs), *Steinernema* spp. and *Heterorhabditis* spp., isolated from different countries against larvae of different mosquito species including *Ae. aegypti* and *Culex pipiens* (Peschiutta et al., 2014; Cagnolo and Almirón, 2017; Chaudhary et al., 2017; Dilipkumar et al., 2018; Tokoz and Saruhan, 2018; Edmunds et al., 2020). These EPNs are lethal insect parasites that vector Gram negative bacteria in the genera *Xenorhabdus* and *Photorhabdus* (Fam: Morganellaceae) into insect hemocoel after gaining entry through natural openings of host; released bacteria produce a combination of enzymes and toxins to overcome host immune system and cause insect death (Shapiro-Ilan et al., 2017, 2020). More recently, Lui et al. (2020) demonstrated that EPNs such as *Steinernema abbasi* can orally invade and puncture the gastric walls of *Ae. albopictus* larvae to access the mosquito hemoceol, but only a few invading IJs can survive melanization and encapsulation to adult emergence. In other studies, the efficacy of the nematode symbiotic bacteria has also been assessed against mosquito larvae (Da Silva et al., 2013; Wagutu et al., 2017; Vitta et al., 2018).

Our study evaluated the influence of water volume and/or depth on the efficacy of different species of EPNs against different larval stages of *Ae. aegypti.* In addition, we compared the effects of cell-free bacterial supernatants and bacterial cell suspensions of the nematode bacterial symbionts and explored their potential for the control of *Ae. aegypti* larvae.

2. Materials and methods

2.1. Maintenance of *Aedes aegypti*

Wild-type adult *Ae. aegypti* were reared at Cardiff School of Biosciences, Cardiff University, Cardiff, UK. Adult mosquitoes were maintained in cages kept in a controlled temperature room at 30 °C with a 12 h light: 12 h dark photoperiod. Mosquitoes were fed every 2 days on defibrinated horse blood (TSC Biosciences Ltd, Buckingham, UK) using an artificial blood feeder (Hemotek membrane feeding systems, Lancs. England). Mosquitoes laid eggs on Whatman 3 MM papers in water-filled cups. Eggs were stored at 4 °C until required or hatched in tap water in small plastic containers (10 × 15 × 15 cm). Eggs hatched within 24 h. Larvae were fed on crushed rabbit pellets and maintained at 22 ± 2 °C. Each larval stage lasted for 2–3 days, thereby, pupal stages were observed 10–14 days after the emergence of the larvae.

2.2. Source of entomopathogenic nematodes-

The EPNs tested included *Heterorhabditis bacteriophora, H. megidis, H. downesi, Steinernema kraussei, S. feltiae* and *S. carpocapsae.* Details of origin and host of each EPN are provided in Table. All nematode species were maintained in final instar larvae of greater wax moth (*Galleria mellonella* L.) as described by Kaya and Stock (1997).

2.3. Screening of entomopathogenic nematodes for the control of Aedes *aegypti larvae*

Screening bioassays were conducted in flat bottom multi dishes ((Nunc^{Tm}, 24-well dishes). Five 3rd instar *Ae. aegypti* were exposed to the following concentrations of EPN infective juveniles (IJ): 0 (control), 10, 250, 500 and 1000 IJs. Each multi dish contained only one treatment and each treatment was replicated 6 times (n = 30). The whole experiment was conducted twice. The final volume of water in the wells was 1.5 mL. The larvae were given 1% final concentration of fish food and incubated at 22 ± 2 °C. Larval mortality was checked daily. Larval feeding on EPN and subsequent melanization and/or growth of EPN was visualized using a Nikon (SMZ 1500) binocular microscope at 10 and 40× magnification. Dead larvae were dissected, and the status of the EPN within the hemocoel was determined microscopically.

Table. Origin, host, and virulence of entomopathogenic species/strains used in this study. Means followed by same lower-case letter are not significantly different from with each other.

Species/strains	Host or source of origin	Virulence (LC_{50}*) IJs/assay
Heterorhabditis bacteriophora UWS1	Soil, *Galleria* bating; grassland, UK	45 ± 0.4^{a}
H. megidis (Larvanem-M)	Koppert Biological Systems, The Netherlands	243 ± 19^{d}
H. downesi	Soil, *Galleria* bating; grassland, Ireland	139 ± 2.5^{b}
Steinernema feltiae (Entonem)	Koppert Biological Systems, The Netherlands	159 ± 9.5^{bc}
S. kraussei	Becker Underwood, Littlehampton, West Sussex, UK	$35\ 5\pm 30^{e}$
S. carpocapsae (Millenium)	Becker Underwood, Littlehampton, West Sussex, UK	93 ± 2.5^{ab}

* IJs number required to cause 50% *Ae. aegypti* larval mortality.

2.4. Influence of EPNs on different larval stages and pupae of *Aedes aegypti*

The susceptibility of the different larval and pupal stages was investigated by exposing 5 *Ae. aegypti* larvae at 1st, 2nd, 3rd, and 4th instar and pupae to a dose of 1000 IJs as described above. Each treatment was replicated 6 times, and the whole experiment was repeated twice. All other experimental conditions were the same as those described above.

2.5. Influence of dead entomopathogenic nematodes on *Aedes aegypti* larvae

To determine if the EPN actively penetrated the larvae or if they were being indiscriminately ingested, five 3rd instar *Ae. aegypti* larvae were exposed to 1000 dead IJs (killed by freezing at –80 °C for 2 h) as described above, and mortality was assessed daily. Controls consisted of live EPN and no EPN.

2.6. Influence of water volume and/or depth on the efficacy of *Heterorhabditis bacteriophora* against *Aedes aegypti*

To determine if the efficacy of the EPN could be reproduced in greater volumes of water, five *Ae. aegypti* larvae were exposed to 1000 IJs of *H. bacteriophora* in 1, 10, 100 and 1000 mL of tap water. Assays were conducted in 24- and 6-well flat bottom multi dishes (Nunc™) for 1- and 5-mL volumes, respectively and in plastic tubs (10 × 15 × 15 cm) for 100 and 1000 mL volumes. Since *Ae. aegypti* larvae are bottom feeders, assays were also conducted at different depths using measuring cylinders, i.e. 100 mL cylinders for 1, 5 and 10 cm depths (with 4, 20 and 40 mL of water, respectively) were used. For 50 cm depth assay, 1-L cylinders with 1.25 L of water (cylinder completely filled with water) were used. For each treatment, a group of five larvae were exposed to 1000 IJs of *H. bacteriophora*. Each treatment was replicated 5 times and the whole experiment replicated twice. All bioassays were conducted at 22 ± 2 °C, and larval mortality was checked daily.

2.7. Larvicidal activity of entomopathogenic nematode symbiont bacteria against *Aedes aegypti*

Larvicidal activities of symbiotic bacteria, *Xenorhabdus nematophila* ATCC 19061 and *Photorhabdus laumondii* TT01, were investigated against *Ae. aegypti* larvae according to Vitta et al. (2018) using cell-free bacterial supernatant (CFS) and bacterial cell suspension. Briefly, CFS and cell suspension were prepared by transferring an overnight culture grown from a single colony on Luria-Bertani agar for 24 h at 28 °C into a fresh LB culture incubated at 28 °C for 72 h. These cultures were then centrifuged at 10.000 rpm at 4 °C for 10 min (Hazir et al., 2016). The supernatants were separated into new Falcon tubes, and the remaining bacterial pellets were re-suspended with sterile physiological saline and turbidity was adjusted to OD_{600nm} = 1.0 using spectrophotometer (Vitta et al., 2018).

In the larvicidal bioassays, third stage larvae were distributed into wells in a 24-well plate (10 larvae/well). Then, different concentrations (final concentrations of 50, 20, 10 and 5%) of the bacterial supernatants and suspensions (10^{7}-10^{8} CFU/mL) were added to wells with 1 mL distilled water. Distilled water alone was used as a negative control. Larvae were fed ground fish food. Each treatment had 10 replicates. The plates were incubated at 26 ± 1 °C. The mortality of the *Aedes* larvae was monitored after 24 and 48 h exposures. Experiments were conducted twice.

2.8. Statistical analysis

The 50% lethal concentration (LC_{50}) and their respective confidence intervals were determined by plotting mortality (0–4 days post-inoculation) vs IJs concentration, using SPSS 16 software (SPSSInc., Chicago, IL, USA). Mortality data were subjected to one-way ANOVA followed by the Tukey test to determine significant difference among the evaluated EPNs. Independent t-test was used to compare the larvicidal effects of cell-free bacterial supernatant and bacterial cell suspension.

3. Results

3.1. Screening bioassay

All EPN species were ingested by *Ae. aegypti* larvae. Mortality was dose dependent with the highest mortality being observed at the 500 and 1000 IJs/well concentrations. At 500 IJ concentration, *H. bacteriophora*, *S. carpocapsae* and *S. feltiae* species caused 16, 13 and 3% larval mortality, respectively at 1-day post inoculation. However, there was only significant difference between *H. bacteriophora* and *H. megidis*, *H. downesi*, *S. kraussei* species (F = 3.849; df = 6.35; P = 0.05) (Figure 1). At 2-day post inoculation, larval mortality increased to 56% for *H. bacteriophora* treatment followed by *S. carpocapsae* (36%) and *S. feltiae* (26%). Except for *S. carpocapsae*, significant difference was observed between *H. bacteriophora* and other nematode species (F= 12.132; df = 6.35; p = 0.001) (Figure 1). Similar results were obtained at 3-day post inoculation. *H. bacteriophora* caused the highest larval mortality (93%), and there was a significant difference between *H. bacteriophora* and other treatments except *S. carpocapsae* (F = 53.500; df = 6.35; p = 0.001) (Figure 1).

At the highest nematode concentration (1000 IJs/well), *H. bacteriophora* and *S. carpocapsae* species exhibited significantly more mortality than the other nematode species at 1-day post inoculation period (F = 19.878; df = 6,35; p = 0.001) (Figure 2). At 2-day post inoculation, *H. bacteriophora* was the most virulence species with 93% larval mortality and there was a significant difference between *H. bacteriophora* and other species (F = 36.667; df = 6.35; p = 0.001) (Figure 2). No larval mortality was observed at control group. At 3-day post inoculation, *H. bacteriophora*, *S. carpocapsae* and *S. feltiae* caused 100, 90 and 66% larval mortality, respectively. *H. bacteriophora* and *S. carpocapsae* species showed statistically more mortality than the other nematode species (F = 41.514; df = 6.35; p = 0.001) (Figure 1).

The EPNs differed significantly in their virulence with *H. bacteriophora* being the most aggressive with an LC_{50} of 45 IJs, almost half that of *S. carpocapsae,* while *H. megidis* and *S. kraussei* had LC_{50} values of 232 and 355 IJs, respectively (Table). None of the mosquito larvae died when exposed to 10 IJs per well (= 2 IJs per larva).

EPNs could be seen within dead *Ae. aegypti* larvae at 2-day post inoculation (Figures 3a, 3b) some of which were melanized (Figure 4). EPN adults and juveniles were observed inside the dead larvae (Figure 3c). Fresh IJs emerged from cadavers 7-day post inoculation (Figure 3c).

3.2. Influence of entomopathogenic nematodes on different larval stages and pupae of *Aedes aegypti*

Significant ($p < 0.05$) differences in larval and pupal susceptibility to EPNs were observed. The 1st and 2nd instar larvae and pupae did not ingest the EPN and consequently showed no sign of infection and survived; however, the 3rd and 4th instars ingested EPN and were highly susceptible. *H. bacteriophora* and *S. carpocapsae* caused 100% mortality, whereas *H. downesi* and *S. feltiae* caused between 40 and 70% mortality of 3rd and 4th instar larvae (data not shown). *H. megidis* and *S. kraussei* were least virulent causing a maximum of 20%–40% mortality. The 3rd and 4th instars did not differ significantly in their susceptibly to the EPNs.

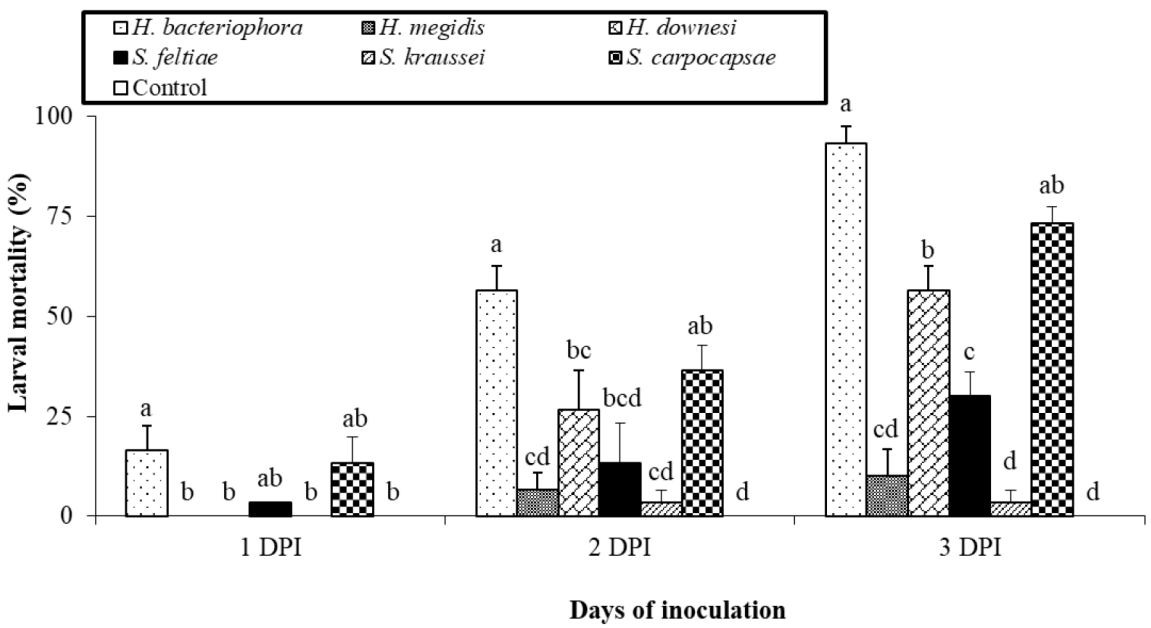

Figure1. Susceptibility of *Aedes aegypti* larvae to different species of EPNs. Five 3rd instar larvae exposed to 500 infective juveniles (IJs) and mortality assessed daily at 1-day, 2- and 3-days post-inoculation (DPI).

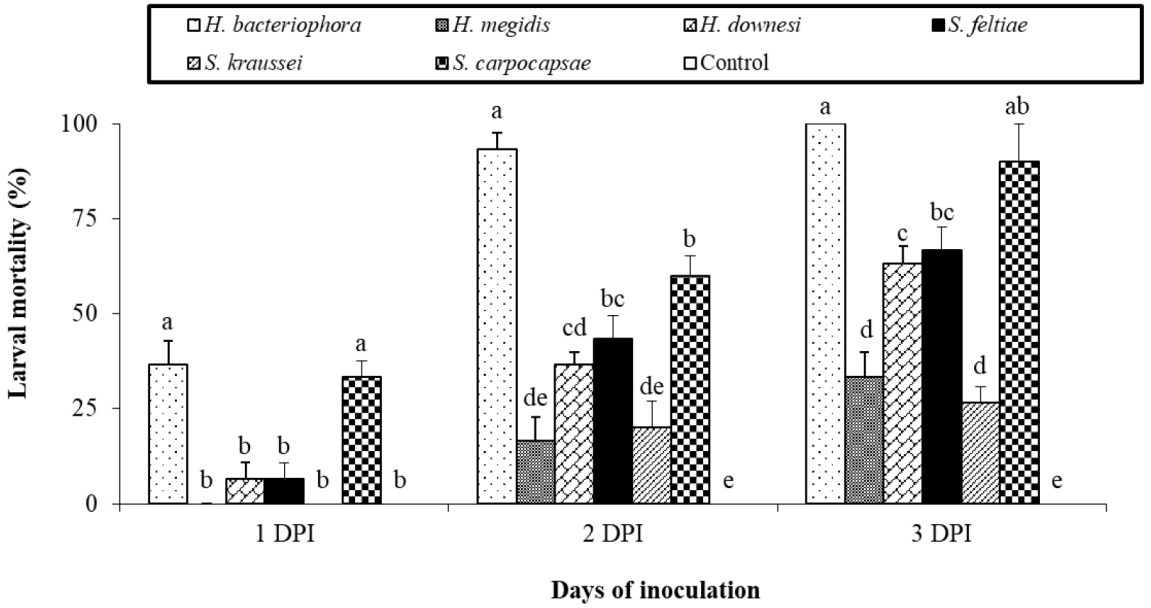

Figure 2. Susceptibility of *Aedes aegypti* larvae to different species of EPN. Five 3rd instar larvae exposed to 1000 infective juveniles (IJs) and mortality assessed daily over 3-day period (DPI).

3.3. Influence of dead entomopathogenic nematodes on *Aedes aegypti* larvae

Dead EPN were ingested by *Ae. aegypti* larvae but caused no mortality. None of the dead EPN was observed in the hemocoel. Similarly, no melanization of dead EPN was observed.

3.4. Influence of water volume and water depth on the efficacy of *Heterorhabditis bacteriophora* against *Aedes aegypti*

The efficacy of *H. bacteriophora* varied significantly only between 10 and 1000 mL water volume at 1-day ($F = 4.121$; $df = 3.16$; $p = 0.024$) and 2-day post inoculation ($F = 5.926$; $df = 3.16$; $p = 0.006$) (Figure 5). At 2-day post inoculation, larval mortality was significantly ($p < 0.05$) lower in 1000 mL (48%) than those exposed to 1–10 mL (70%–80%). However, at 3-day post inoculation, mortality at all water volumes varied non-significantly ($F = 0.667$; $df = 3.16$; $p = 0.585$) (Figure 5).

Regarding with water depth, no significant difference was observed among the water depths at any day of post inoculation ($p > 0.05$) (Figure 6).

3.5. Larvicidal activity of entomopathogenic nematode symbiont bacteria against *Aedes aegypti*

After 24 h, a downward trend was observed in the mortality rate of *Ae. aegypti* after exposure to different concentrations of *X. nematophila* supernatant ($F = 56.89$; $df = 4$; $p < 0.001$) and cell suspension ($F = 202.07$; $df = 4$; $p < 0.001$), with the highest mortality rate occurring in treatments with 50% concentration. Both 50% and 20% concentrations caused >91% larval mortality after 48 h and there was no significant difference between these concentrations. However, statistically significant difference occurred between these groups and the other treatments including supernatant and cell suspension ($p < 0.001$) (Figure7).

In the case of *P. laumondii,* the bacterial supernatant was not effective against mosquito larvae at all tested concentrations, but cell suspensions had a significant effect compared to the control after 24 and 48 h exposure. Fifty percent and 20% bacterial suspensions caused 75 and 63% larval mortality, respectively, while 10 and 5% concentrations killed 28%–13% of treated larvae ($F = 38.93$; $df = 4$; $p < 0.001$) after 24 h. After 48 h, a slight increase in the larval mortality rates was observed in all the treated groups. There was a significant difference between 50 and 20% concentration and the other treatments and control ($F = 85.96$; $df = 4$; $p < 0.001$) (Figure 7).

According to independent t-test, there was no statistical difference between the CFS and bacterial cell suspension of *X. nematophila* at any concentration ($p > 0.05$). However, in *P. laumondii*, significantly more larval mortality was observed in bacterial cell suspension treatments than CFS at all tested concentrations ($p < 0.05$).

4. Discussion

This study shows that older (3rd and 4th) *Ae. aegypti* larvae will readily ingest EPN, which ultimately leads to their death. Early (1st and 2nd) instars did not ingest any EPN and, thus, escaped infection, presumably due to their small mouthparts or feeding behavior that excludes large particles. Several researchers have assessed the biocontrol potential of

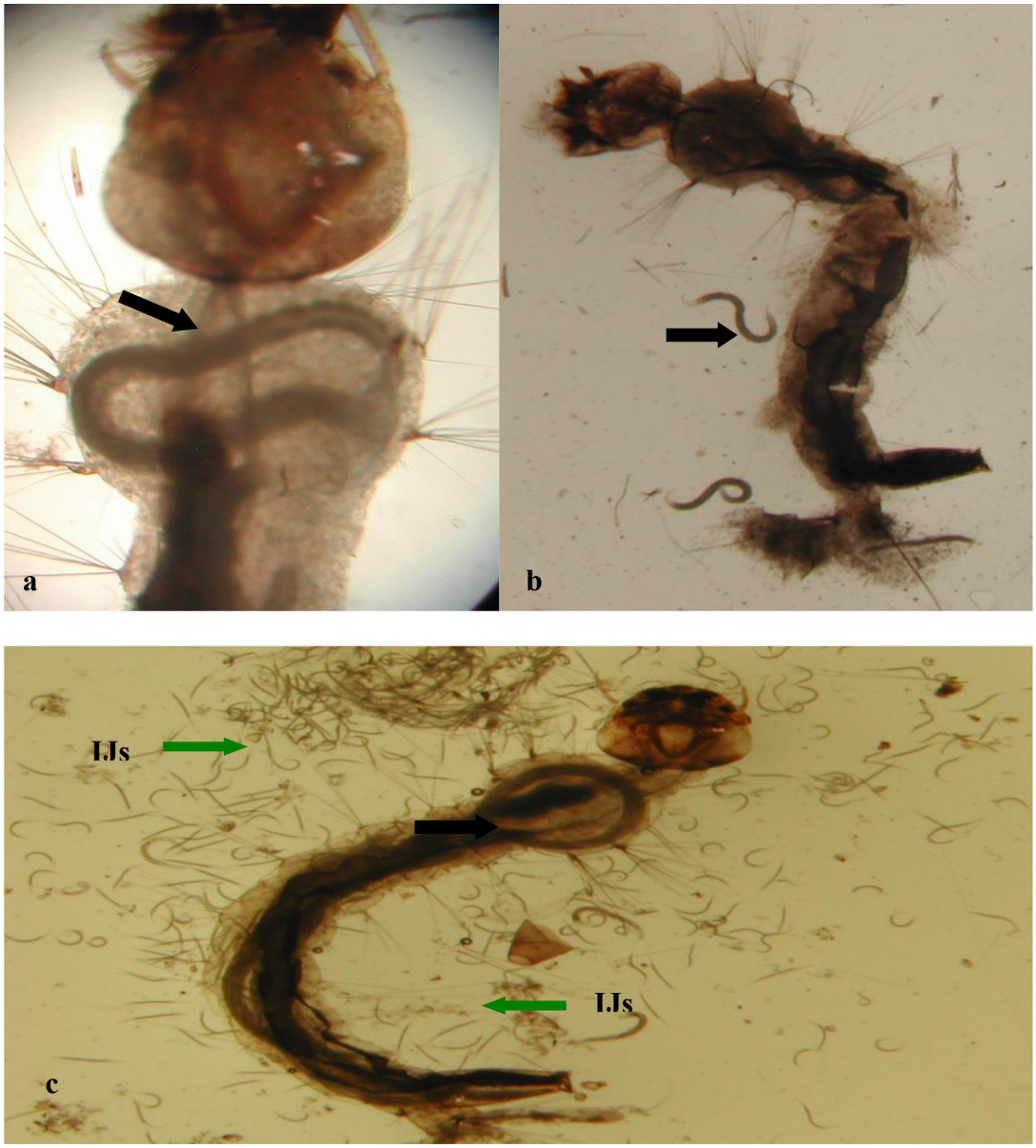

Figure 3. Different stages of *Heterorhabditis bacteriophora* colonization of *Aedes aegypti* larvae (3rd instar). *H. bacteriophora* within larvae at 2-day post inoculation (a), *H. bacteriophora* emerging out of larvae upon larval dissection at 7-day post inoculation) (b), adult *H. bacteriophora* within larvae along with large number of infective juveniles (IJs) released from another adult *H. bacteriophora* (c). Black arrows indicate adult *H. bacteriophora,* whereas green arrows indicate newly emerged IJs.

different EPN species on different larval stages of mosquitoes. For example, Poinar and Kaul (1982) also reported that *H. bacteriophora* preferentially infected older instar *Culex* species. Likewise, de Oliveira Cardosoa et al. (2015) demonstrated that *H. baujardi* and two strains of *H. indica* were not virulent against L1 and L2 instars of *Ae. aegypti* and that only L3 and L4 were affected by these EPN species. The fact that EPNs may only be infecting older instar mosquitoes does not preclude their use in pest control programs as they are preventing adult emergence, and subsequently this will lead to a reduction in mosquito populations. The feeding is indiscriminate because larvae consume different species of EPN as well as dead and live EPNs. Our studies also showed that larval mortality was dose dependent with greater and more rapid mortality caused by higher EPN concentrations as indicated in previous experiments (Molta and Hominick, 1989; Poinar and Kaul, 1982; Cagnolo and Almirón, 2017; Chaudhary et al., 2017; Aiswarya et al., 2018). These findings

Figure 4. Melanization of *Heterorhabditis bacteriophora* within *Aedes aegypti* larvae (3rd instar). A melanized *H. bacteriophora* within dead *Ae. aegypti* larvae (a), close up picture of melanized nematode upon larval dissection (b), nematodes representing different stages of melanization recovered from one dead *Ae. aegypti* larvae (c). Arrows indicate melanized nematode within *Ae. aegypti* larvae.

are in line with those reported for other insects or insect life stages commonly found in terrestrial habitats (Georgis et al., 2006). Chaudhary et al. (2017) investigated the effects of *S. kraussei* and *H. bacteriophora* against *Ae. aegypti* at different concentrations and temperature conditions in canal, sewage and tap water and reported that both EPN species were highly effective against the larvae, inducing 100% larval mortality optimally at 20 °C and 30 °C.

EPNs appear to work at different depths and volumes of water similar to those found in *Aedes* breeding sites such as drums, jars concrete tanks and discarded objects (Phong and Nam, 1999). Water volume or depth had minimal or no impact on overall mortality of *Ae. aegypti* exposed to *H. bacteriophora*, particularly at later time points. In these experiments, a fixed number of IJs were added to a variable water volume. At 4 days post-inoculation, there

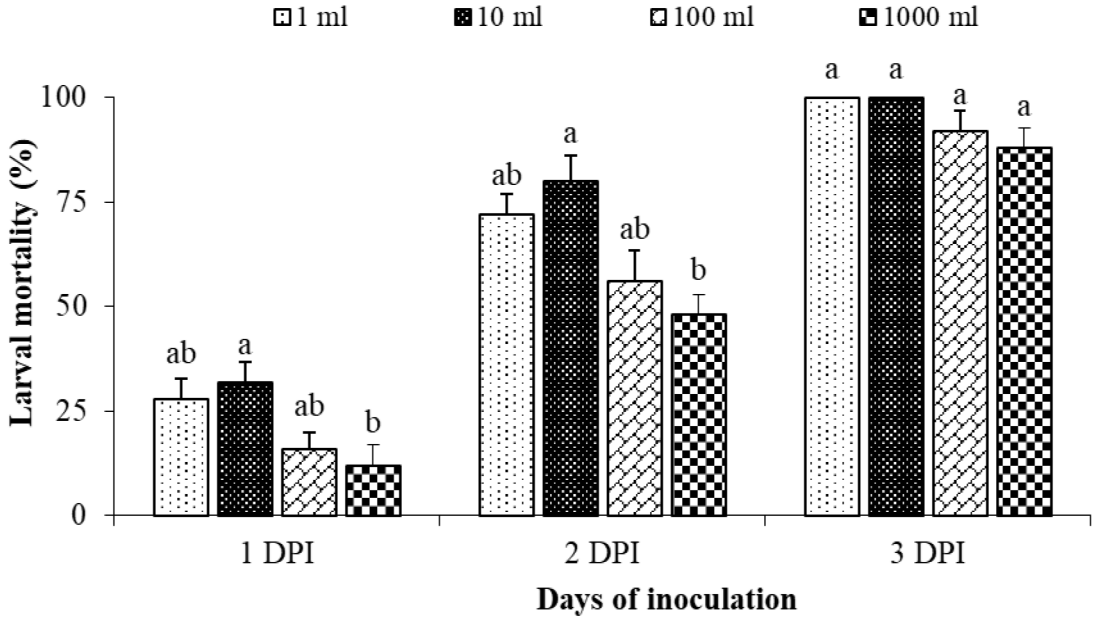

Figure 5. *Aedes aegypti* larval mortality when exposed to 1000 infective juveniles (IJs) of *Heterorhabditis bacteriophora* in different volumes of water.

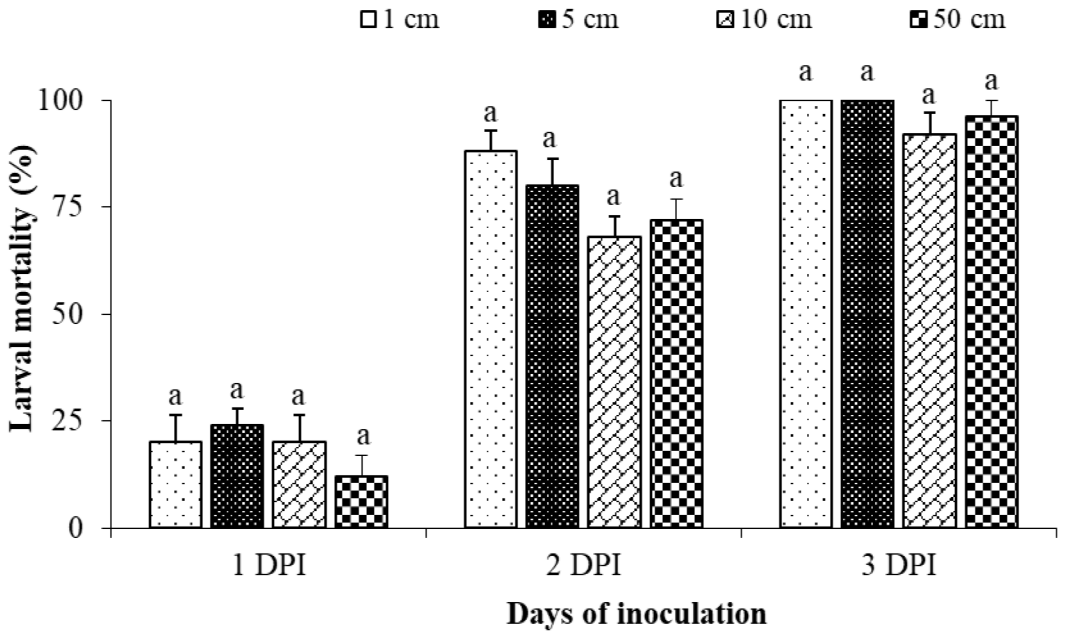

Figure 6. *Aedes aegypti* larval mortality when exposed to 1000 infective juveniles (IJs) of *Heterorhabditis bacteriophora* at different depths of water.

were no significant differences in mortality, which may suggest that after this period, despite the greater volumes and consequently lower IJ densities, sufficient IJs had been ingested to reach the mortality threshold. This, again, illustrates the suitability of EPN applications to *Ae. aegypti* breeding site. It should be emphasized that our study only assessed the effects of water volume and depth on EPN survival after 3 days, which is relatively short. Oxygen deprivation might affect EPN survival over longer periods of time; hence, studies in aquatic conditions in the field are needed.

Although all EPNs caused some mortality, *H. bacteriophora* was the most aggressive suggesting that it is better adapted for infecting *Ae. aegypti* larvae. The less

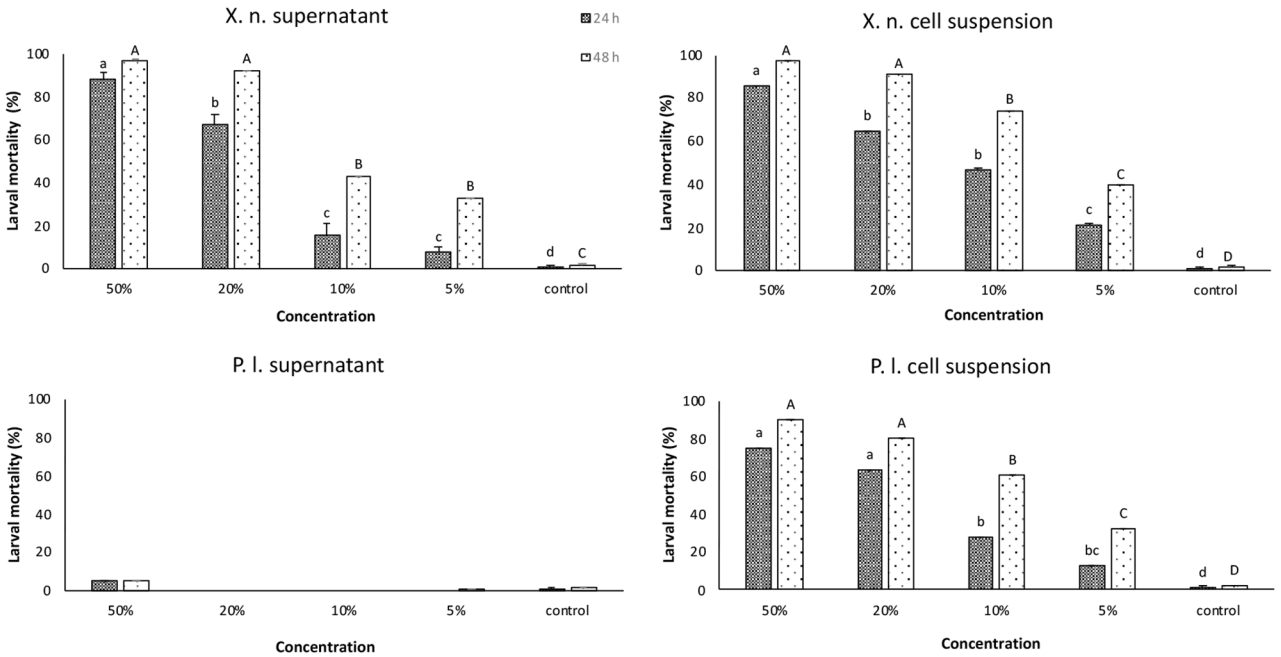

Figure 7. *Aedes aegypti* larval mortality when exposed to supernatants and cell suspensions of *Xenorhabdus nematophila* (X. n.) and *Photorhabdus laumondii* (P. l.) in 24 well plates. Different uppercase or lower letters above error bars indicate statistical significance (Tukey's test $p \leq 0.05$).

pathogenic EPNs may have been less effective at penetrating the gut and accessing the nutrient rich hemolymph. Wang et al. (1995) reported that up to 40% EPN are killed in the gut of the coleopteran *Popillia japonica*. Since host suitability is dependent on the ability of the parasite to evade or suppress the insect immune responses (Wang et al., 1995; Peters and Ehlers, 1997), it is possible that virulent species, i.e. *H. bacteriophora* and *S. carpocapsae* were able to suppress host defenses more quickly than other species. When penetrating the insect's hemocoel, the IJs encounter the non-self-response by the immune system of the host. For example, melanization, a humoral immune response related to the production of reactive oxygen species, can kill invaders (Nappi and Vass, 2001). However, these responses can be suppressed by the release of bacteria associated with EPNs (Forst et al., 1997; Owuama, 2001). Indeed, inability of dead EPN to cause any mortality or elicit host defenses clearly suggests that larval mortality and/or EPN melanization was triggered by penetration into the hemocoel. Once inside the hemolymph, the EPN symbiotic bacteria have to contend with the hosts' defenses (Wang et al., 1995; Peters and Ehlers, 1997), and the *H. bacteriophora* symbiotic bacteria may be more pathogenic than those of the other EPN species.

In our study, mosquito larvae continue to ingest EPNs; therefore, their defenses appeared to be overwhelmed with EPN and/or their associated bacteria and EPN ingested later may have avoided larval responses and, therefore, caused successful infection. The fact that none of the larvae died at lower concentrations of 2–50 EPN/larvae (except for *H. bacteriophora*) further confirms that the larvae were able to defend themselves at lower EPN concentrations; however, at numbers as high as 100/larvae, EPN concentration proved to be fatal for larvae. Survival of 3–8 mature EPNs in each dead larva further suggests that a large number of ingested EPN failed to survive; however, their interaction with host defenses provided an opportunity for other individuals to cause mortality. Similarly, Lui et al. (2020) reported that although invading *S. abbasi* IJs were melanized and encapsulated by *A. albopictus* immune system, larger infection numbers allowed a few nematode juveniles to survive till adult emergence. The death of a proportion of EPN was also observed by Poinar and Kaul (1982) with *H. bacteriophora* in *Cx. pipiens* larvae. However, these authors found no survival of nematodes after the death of the mosquito larvae, whereas we showed in *Ae. aegypti* that all EPNs can emerge from larval cadavers as indicated previously in a research on *S. carpocapsae* conducted by Welch and Bronskill (1962). This may indicate that *Ae. aegypti* is a better target for control with EPNs than *Culex* species.

We also assessed the larvicidal effects of the CFS and bacterial cell suspension of *P. laumondii* and *X. nematophila,* bacterial symbionts of *H. bacteriophora* and *S. carpocapsae,* respectively. Our results showed that both the bacterial supernatant and bacterial suspensions of

X. nematophila caused >91% larval mortality after 48 h, whereas only the bacterial cell suspension of *P. laumondii* was effective against the mosquito larvae. These data suggest that some bacteria like *X. nematophila* release toxic metabolic compounds with larvicidal activities out of their cells compared to *P. laumondi* supernatant, which was ineffective against the mosquito larvae. Da Silva et al. (2013) demonstrated that 73%–83% of *Ae. aegypti* larvae dosed with *P. luminescens* bacterial cell suspension died, whereas *X. nematophila* killed 52% of fed larvae and 42% of unfed larvae in their fed-unfed treatments. Vitta et al. (2018) assessed the effects of bacterial cell suspensions of *X. stockiae, X. indica, P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis* isolated from Thailand on both *Ae. aegypti* and *Ae. albopictus*. The authors observed larval mortality between 66 and 73% for *Ae. aegypti* and 36 and 77% for *Ae. albopictus* 48 h after treatment.

Following a successful infection of an insect host by EPNs, hundreds of thousands of IJs emerge and leave in search of new hosts, carrying with them an inoculation of mutualistic bacteria received from the internal host environment (Boemare, 2002). In water, IJs can survive over 6 months even at temperatures of 20–25 °C (Poinar and Kaul, 1982); therefore, EPNs could provide a sustained control of *Ae. aegypti* over a long period of time. The fact that virulent species of EPNs identified in this study are known to work better at higher temperatures, i.e. 20–30 °C (Kim and Alston, 2008; Power et al., 2009) makes their application in *Ae. aegypti* hot spots even more practicable and promising. Future studies should also endeavor to identify which of the array of bioactive larvicidal compounds produced by EPN symbiotic bacteria are responsible for the activity. Overall, this study provides an opportunity to devise a novel strategy based on virulent EPN species to control larval stages of *Ae. aegypti*. Application of EPN to *Ae. aegypti* breeding sites could help reduce *Ae. aegypti* populations and hence reduce the risks of future outbreaks of Chikungunya, dengue and yellow fever.

Conflict of interest

Authors declare that they have no conflict of interest.

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