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In vitro evaluation of the effect of entomopathogenic nematodes on different developmental stages of the brown dog tick *Rhipicephalus sanguineus* (Acari: Ixodidae)

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Original research

ABSTRACT

The brown dog tick, *Rhipicephalus sanguineus* (*R. sanguineus*) is a worldwide ectoparasite of veterinary and medical concern. Entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis* are occasionally used as bio-control agents for external parasites such as ticks and flies. This study aimed to evaluate the effect of five different concentrations of the EPNs *Heterorhabditis bacteriophora* HP88 and *Steinernema* sp. SII [4000, 2000, 1000, 500, and 250 infective juveniles per milliliter (IJs/ml)] on different developmental stages of *R. sanguineus* ticks. The EPN application caused mortality in both engorged larvae and newly molted nymphs [*H. bacteriophora* HP88 (10% - 40%) and *Steinernema* sp. SII (10% - 18%)]. However, mortality was much higher in both engorged nymphs and newly molted adults (54% - 82% with *H. bacteriophora* HP88 and 84% - 96% with *Steinernema* sp. SII). Both EPNs were 100% effective on engorged females 9 days post-exposure. As this life stage was the most susceptible, it was used to calculate the LC (lethal concentration) values in order to determine which EPN was most virulent. The LC₅₀ and LC₉₅ at 5 days post-exposure were 76 and 5421 IJs/ml for *H. bacteriophora* HP88 and 885 and 5700 IJs/ml for *Steinernema* sp. SII, respectively. In conclusion, although the efficacy of the two EPN species differed among tick life stages, both nematode species can induce mortality and could be used as an alternative option for the biological control of non-parasitic (off-host) tick stages.

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Keywords biological control; ectoparasite; *Heterorhabditis*; *Steinernema*; medical and veterinary entomology

Introduction

The brown dog tick, *Rhipicephalus sanguineus* (*R. sanguineus*) (Latreille, 1806) (Acari: Ixodidae), is an important globally distributed ectoparasite of veterinary and human medical concern (Dantas-Torres 2010). It is a three-host tick and requires three blood meals to complete its life cycle (Walker *et al.* 2003). This tick species parasitizes mainly dogs and other carnivores, as well as sporadically infesting livestock, wild ruminants, rodents and humans (Dantas-Torres

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2010; Gray *et al.* 2013; Mentz *et al.* 2016; Didyk *et al.* 2022). It causes tissue damage, anemia, and enhances conditions for secondary bacterial infections and myiasis (Jongejan and Uilenberg 2004; Lew-Tabor and Rodriguez Valle 2016). This tick species has the particularity of being able to survive indoors and can undergo rapid population increases in a short period of time resulting in severe infestations (Uspensky and Ioffe-Uspensky 2002). Mature and immature stages of *R. sanguineus* are considered vectors for diseases caused by bacteria (*Rickettsia* (*R. rickettsii*, *R. conorii*, *Ehrlichia canis*, *Coxiella burnettii*), protozoa (*Babesia* (*B. canis*, *B. gibsoni*, *Hepatozoon canis*) and viruses (Crimean-Congo hemorrhagic fever) that pose a serious threat to public and animal health (Parola *et al.* 2009; Tahmasebi *et al.* 2010; Dantas-Torres 2010; Dantas-Torres and Otranto 2015; Silva *et al.* 2017).

Until now, chemical acaricide use during the parasitic phase has been the most common and efficient method to control ticks in livestock production (Aquino-Bolanos *et al.* 2019). However, the excessive use of chemical acaricides has produced serious complications, including multi acaricide -resistance in ticks (Reck *et al.* 2014; Klafke *et al.* 2017), toxicity to mammals, and pollution of the environment (Amaral *et al.* 2011; Gomes *et al.* 2011). Therefore, the use of alternative and safer control methods are needed to control ticks. Biological control using entomopathogenic nematodes (EPNs) is a promising alternative option for tick control (Goolsby *et al.* 2018).

Investigations have revealed that EPNs are lethal to many insect pests and some important parasites such as ticks (Samish and Glazer 1991; Monteiro *et al.* 2014; Butt *et al.* 2016; Goolsby and Shapiro-Ilan 2020). In particular, *Steinernema* and *Heterorhabditis* nematodes are engaged in mutualistic associations with the motile Gram-negative bacteria *Xenorhabdus* and *Photorhabdus*, respectively (Goodrich-Blair and Clarke 2007; El-Sadawy *et al.* 2016); these bacteria are released into the arthropod host after infection by the nematode and can be toxic for the host. *Heterorhabditis bacteriophora*, *H. baujardi*, *H. indica*, *H. floridensis*, *Steinernema* sp. SII, *S. riobrave* and *S. feltiae* were all found to be virulent for the cattle tick *Rhipicephalus microplus* (Canestrini, 1888) (Goolsby *et al.* 2018; de Mendonça *et al.* 2019; Singh *et al.* 2019; Goolsby and Shapiro-Ilan 2020; Monteiro *et al.* 2020). Moreover, *Steinernema* sp. SII exhibited a pathogenic effect against adults and nymphs of the tick *Hyalomma anatolicum excavatum* Koch, 1844 (Butt *et al.* 2016) and *H. bacteriophora* and *H. indica* were shown to negatively affect the reproductive biology of partially engorged females of *Dermacentor nitens* Neumann, 1897 (Monteiro *et al.* 2014).

Samish *et al.* (1999) evaluated the susceptibility of larval, nymphal, and adult stages of the tick *R. sanguineus* and *Hyalomma excavatum* to two strains of *Steinernema carpocapsae* and three strains of *Heterorhabditis bacteriophora*. They found that the adult ticks were more susceptible than larvae and nymphs to these EPNs. Both larvae and nymphs of *R. sanguineus* showed poor susceptibility with average respective mortalities of 15% and 14% across nematode species. Engorged females were most susceptible and showed an average mortality of 93.2% at 5,000 nematodes per dish across nematode strains; this was higher than for engorged males (73.2%). No information about the effect of EPNs on newly molted *R. sanguineus* ticks when exposure occurs in the engorged state is currently available. Here, we retested the effect of *H. bacteriophora* HP88 and a new species of *Steinernema* sp. SII on different developmental stages of *R. sanguineus*, including newly molted nymphal and adult ticks, to evaluate their potential use in the biological control of this tick species

Material and methods

Ethical approval

This study was approved by Medical Research Ethics Committee (MREC) at the National Research Centre (NRC), Egypt in accordance with local laws and regulations (approval protocol No 1550212023).

Ticks

Rhipicephalus sanguineus engorged females were obtained from an established colony (started in February 2021) at the Parasitology and Animal Diseases Department, Veterinary Research Institute, National Research Centre, Dokki, Giza, Egypt. This established colony started with a limited number of females that were collected from a single dog and were initially identified according to Walker *et al.* (2003). These females were placed in an incubator (Friocell, MMM, Germany) in plastic cups for oviposition ($25\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$ and 75%–80% relative humidity) and eggs were incubated for hatching. The hatched larvae from different females were mixed and were allowed to feed on healthy rabbits using the capsule technique to obtain engorged larvae (El Hakim *et al.* 2011). Specifically, the ticks were placed inside a feeding capsule consisting of a plastic tube (2.5 cm in diameter and 3 cm high) glued to the shaved back of the rabbit (two capsules per animal). Wooden collars (designed from 1 mm thick plywood) were placed on the rabbits to prevent grooming (Abdel-Shafy 2008). A portion of the engorged larvae were used for the treatments and others were incubated for the nymphal molt. Molted nymphs were fed on healthy rabbits to obtain fully engorged nymphs. Again, a portion of the engorged nymphs were used in treatments and others were incubated to obtain unfed adults. An equal number of unfed males and females were fed on rabbits to obtain fully engorged females. Males were included in the feeding step because females cannot reach full engorgement without mating.

Entomopathogenic nematodes

The infective juvenile larvae (IJs) of entomopathogenic nematodes live in the upper layer of the soil and find their hosts by either searching (cruisers) or by waiting (ambush); they are attracted to the host's bio-secretions. Once the IJs find their host, they invade it through the host's natural body openings such as the genital pore, anus, spiracle, mouth parts or, occasionally, via the cuticle (Kocan *et al.* 1998). The IJs then migrate to the host haemocoel and release bacteria, which are able to evade the insect's immune response and kill it using a variety of toxic mechanisms (Bowen and Ensign 1998; Morgan *et al.* 2001).

Two species of EPNs, *Heterorhabditis bacteriophora* HP88, and *Steinernema* sp. SII, were propagated *in-vivo* on 6th instar larvae of the greater wax moth *Galleria mellonella* according to Dutky *et al.* (1964) or *in-vitro* on solid culture according to El-Sadawy (2011). These experiments were carried out at the Parasitology and Animal Diseases Department, National Research Centre.

Investigation of entomopathogenic nematode virulence on different developmental stages of *R. sanguineus*

The effect of *H. bacteriophora* HP88 and *Steinernema* sp. SII on *R. sanguineus* (engorged larvae, engorged nymphs, and engorged females) were evaluated. EPNs were suspended in tap water at concentrations of 4000, 2000, 1000, 500, and 250 infective juveniles per milliliter (IJs/mL). These concentrations were applied 4 days after engorgement. Plastic cups (3 cm diameter x 3 cm long) containing 10 g of fine sand were prepared for this experiment, with 5 ticks per cup for engorged adults, 10 ticks/cup for engorged larvae, and 10 ticks/cup for engorged nymphs. Two mL of the suspended IJs was directly added to each cup, with the 5 treatments replicated 5 times for each life stage (total of 75 cups). Five additional control replicates were also prepared for each stage using tap water free from IJs. All cups were covered with muslin cloth and left under room conditions ($25\text{--}30\text{ }^{\circ}\text{C}$, 14h:10h light–dark) at the same time for all tick stages. Different cycles of *R. sanguineus* were initiated from the first generation at different times. In this way, all tick stages were available at the same time for experimentation. All cups were examined daily post-treatment for 11 days for engorged larvae, 24 days for engorged nymphs, and 10 days for fully engorged females.

The mortality of tick stages was recorded based on their appearance using the specific symptom of EPN infection, that is darkened swollen bodies. Other additional symptoms, such

as no response to stimulation in case of active tick stages, was also used. Tick mortality was recorded starting from the 6th, 10th and 1st day post EPN exposure in engorged larvae, engorged nymphs, and fully engorged females, respectively.

Statistical analysis

The percent mortality of engorged larvae, engorged nymphs and engorged females exposed to entomopathogenic nematode species was statistically analyzed by one-way ANOVA tests, followed by Tukey tests using SPSS program version 20. The LC₅₀ and LC₉₅ values were calculated by applying regression equation analyses to the probit-transformed mortality of engorged females. The dose-response data were analyzed by the probit method (Finney 1962) using Ldp line[®] software (Ehabsoft).

Results

Virulence of EPN species on engorged larvae

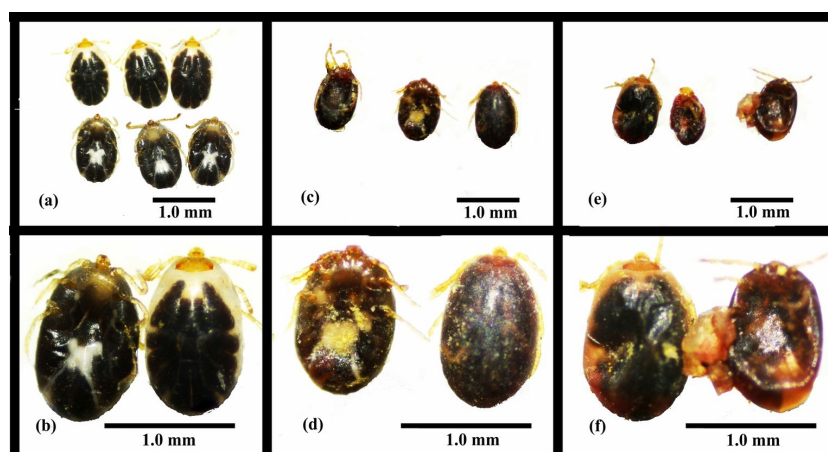


Figure 1 Engorged larvae of *Rhipicephalus sanguineus* 6 days post-exposure to infective juvenile (IJs) of entomopathogenic nematodes: a – larvae in the control group. b – zoom in from a. c – larvae infected with *Heterorhabditis bacteriophora* HP88. d – zoom in from c. e – larvae infected with *Steinernema* sp. SII. f – zoom in from (e).

Engorged larvae showed some resistance against the tested EPNs. The impact of the two EPNs on engorged larvae extended to the molted nymphs as the EPNs remained alive in the moist sand and were able to infect the newly molted nymphs. Engorged larvae molted to the nymphal stage after approximately one week. The mortality of engorged larvae and newly molted nymphs was recorded at the end of the experimental observation time (11 days). *Heterorhabditis bacteriophora* HP88 and *Steinernema* sp. SII caused mortality ranging from 10% to 40% and from 10% to 18%, respectively, in engorged larvae and newly molted nymphs (Table 1). A significant effect of EPN dose was seen for *H. bacteriophora* HP88 starting at 2000 IJs/mL, whereas there was no dose effect for *Steinernema* sp. SII (Table 1). Dead larvae were observed to have swollen bodies and darkened color starting from the 6th day post-exposure to both EPNs (Figure 1). The dead exposed nymphs also seemed darker than controls.

Virulence of EPN species on engorged nymphs

The engorged nymphs were more susceptible to nematode infections than engorged larvae. As for the engorged larvae, the EPN treatment extended to the newly molted unfed adult due to the

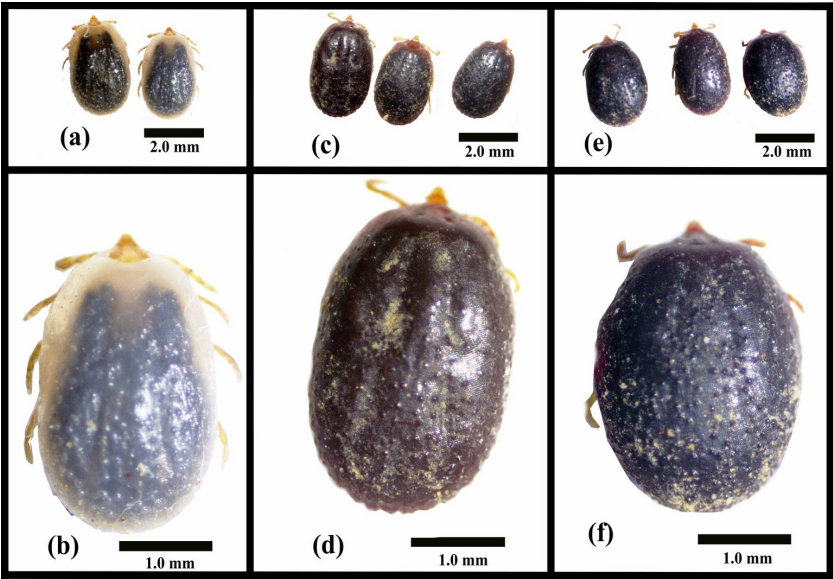


Figure 2 Engorged nymphs of *Rhipicephalus sanguineus* 10 days post-exposure to infective juvenile (IJs) of entomopathogenic nematodes: a – nymphs in the control group. b – zoom in from a. c – nymphs infected with *Heterorhabditis bacteriophora* HP88. d – zoom in from c. e – nymphs infected with *Steinernema* sp. SII. f – zoom in from e.

presence of EPNs in the moist sand. The molting of engorged nymphs to adults occurred after 12-14 days. The mortality of both engorged nymphs and newly molted adults was recorded at the end of the experimental observation period (24 days). In general, *H. bacteriophora* HP88 and *Stienernema* sp. SII caused mortality ranging from 54% to 82% and from 84% to 96%, respectively, in engorged nymphs and molted adults (Table 2). A significant effect of EPN dose was seen for *H. bacteriophora* HP88 and *Steinernema* sp. SII compared with controls (Table 2). *Steinernema* sp. SII tended to show higher virulence than *H. bacteriophora* HP88, except for concentrations at 2000 and 1000 IJs/mL (Table 2). The optimum EPN concentrations for both strains were between 1000 and 2000 IJs. Dead nymphs exposed to *H. bacteriophora* HP88 were distinguishable by a reddish color, while dead nymphs exposed to *Stienernema* sp. SII tended to go black (Figure 2). The dead newly molted adults, including both males and females, were darker in color than surviving adults for both EPNs and this symptom was observed starting on the 13th day post-exposure.

Table 1 Percent mortality (mean ± SE) of engorged larvae and newly molted nymphs of *Rhipicephalus sanguineus* 11 days post-exposure to entomopathogenic nematodes, *Heterorhabditis bacteriophora* HP88 and *Steinernema* sp. SII. The results of the one-way ANOVA test for each EPN species is given in the last two columns and that between EPNs in the last two rows. Significant P values are in bold. Capital letters in the same row indicate significant differences between means according to a Tukey’s test at P<0.05.

Nematode species	Concentration (IJs/ml)						F _(5, 24)	P value
	4000	2000	1000	500	250	Control		
<i>Heterorhabditis bacteriophora</i> HP88	40±7.1 ^A	22±5.8 ^{AB}	8.0±5.8 ^{BC}	10±4.5 ^{BC}	10±3.2 ^{BC}	0±0 ^C	8.092	<0.001
<i>Steinernema</i> sp. SII	18±9.2	12±3.7	10±6.3	10±5.5	10±6.3	0±0	0.969	0.456
F _(1, 8)	3.612	2.083	0.054	0	0			
P value	0.096	0.187	0.822	1	1			

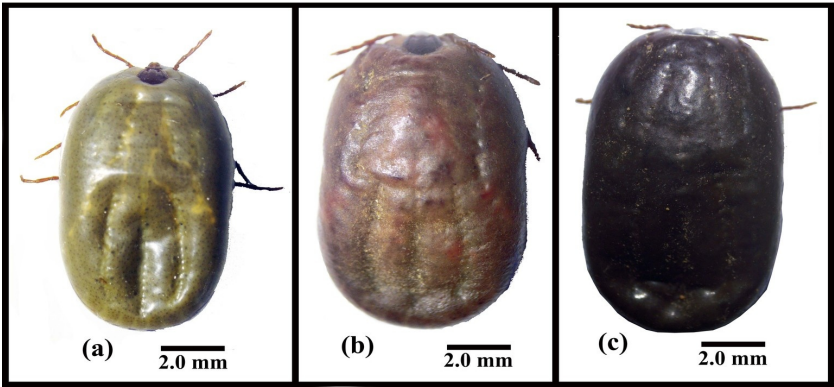


Figure 3 Engorged females of *Rhipicephalus sanguineus* exposed to infective juvenile (IJs) of entomopathogenic nematodes: a – female in the control group. b – female infected with *Heterorhabditis bacteriophora* HP88. c – female infected with *Steinernema* sp. SII.

Virulence of EPN species on fully engorged females

The mortality of fully engorged females was observed starting from the 1st day post-exposure with both EPN species and increased gradually to reach 100% on the 9th day post-infection for all concentrations, except 250 IJs of *Steinernema* sp. SII which remained at 84% mortality (Table 3). The LC₅₀ and LC₉₅ on the 5th day post-exposure were 76 and 5421 IJs for *H. bacteriophora* HP88 and 885 and 5700 IJs for *Steinernema* sp. SII, respectively (Table 4). Although *H. bacteriophora* HP88 seemed more pathogenic than *Steinernema* sp. SII against engorged females, these differences were not statistically significant. A significant effect of EPN dose was seen for *H. bacteriophora* HP88 and *Steinernema* sp. SII compared to controls, whereas there was no EPN species effect (Table 3). Dead females exposed to *H. bacteriophora* HP88 had reddish color, while those exposed to *Steinernema* sp. SII had a black color similar to that observed in engorged nymphs (Figure 3).

Discussion

Most studies that test the use of nematodes in biological control against ticks were performed in the laboratory on adult stages. Few studies have evaluated the impact of nematodes on immature stages (Hill 1998; Samish *et al.* 1999). This study aimed to evaluate the effect of two nematode species *Heterorhabditis bacteriophora* HP88 and *Steinernema* sp. SII of two different families (Heterorhabditidae and Steinernematidae) on all tick life stages, including the subsequent newly molted ticks. We also used cups filled with moist sandy soil in order to mimic the natural habitat in which these feeding stages typically exist. We believe this evaluation

Table 2 Percent mortality (mean ± SE) of engorged nymphs and newly molted adults of *Rhipicephalus sanguineus* after 24 days post-exposure to entomopathogenic nematodes; *Heterorhabditis bacteriophora* HP88 and *Steinernema* sp. SII. The results of the one-way ANOVA test for each EPN species is given in the last two columns and that between EPNs in the last two rows. Significant P values are in bold. Capital letters in the same row indicate the significant differences between means corresponding to concentrations according to a Tukey’s test at P<0.05.

Nematode species	Concentration (IJs/ml)						F _(5,24)	P value
	4000	2000	1000	500	250	Control		
<i>Heterorhabditis bacteriophora</i> HP88	68±5.9 ^A	82±3.7 ^A	72±13.6 ^A	56±12.1 ^A	54±5.1 ^A	0±00 ^B	12.515	<0.001
<i>Steinernema</i> sp. SII.	88±4.8 ^A	90±3.1 ^A	96±2.4 ^A	90±4.4 ^A	84±4.0 ^A	00.00 ^B	106.821	<0.001
F _(1,8)	6.897	2.667	3.032	6.964	21.429	-		
P value	0.03	0.141	0.12	0.03	0.002	-		

Table 3 Percent mortality (mean±SE) of engorged females of *Rhipicephalus sanguineus* 9 days post-exposure to entomopathogenic nematodes *Heterorhabditis bacteriophora* HP88 and *Steinernema* sp. SII. The results of the one-way ANOVA test for each EPN species is given in the last two columns and that between EPNs in the last two rows. Significant P values are in bold. Capital letters in the same row indicate the significant differences between means corresponding to concentrations according to a Tukey's test at $P < 0.05$.

Nematode species	Concentration (IJs/ml)						F _(5,24)	P value
	4000	2000	1000	500	250	Control		
<i>Heterorhabditis bacteriophora</i> HP88	100	100	100	100	100	0	-	-
<i>Steinernema</i> sp. SII	100 ^A	100 ^A	100 ^A	100 ^A	84±7.5 ^B	0 ^C	171.714	<0.001
F _(1,8)	-	-	-	-	4.571	-		
P value	-	-	-	-	0.065	-		

provides a more complete picture of the potential impact of EPNs as biological control agents.

The mortality of newly molted adults and engorged females was observed earlier than in the other tick stages despite having larger bodies. This might be attributed to these phases possessing large and more numerous orifices than juvenile stages which would allow a large number of IJs to enter the body cavity and release a greater quantity of symbiotic bacteria (Forst *et al.* 1997). Reddish swollen bodies in engorged females and engorged nymphs were observed in case of *H. bacteriophora* HP88 infection, while blackened swollen bodies of these stages corresponded to *Steinernema* sp. SII infection. These findings are similar to those of Woodring and Kaya (1988) who identified EPN genera based on the color of cadavers of the wax moth, *Galleria mellonella* L. in which *Heterorhabditis* and *Steinernema* produce red and tan colors, respectively. However, in the present study, it was difficult to discriminate infection by these two EPN genera in engorged larvae because all dead larvae were black in color.

We found that *R. sanguineus* tick larvae were the least susceptible stage to EPN infection, with the percent mortality being no more than 8%. Hill (1998) found that EPNs were unable to completely infect engorged larvae of the tick *Ixodes scapularis* Say, 1821; this result may be due to the nature of the tick species, nematode species, the environment, and/or the methodology of the experiment. In contrast, Samish *et al.* (1999) reported that engorged larvae of the tick species *R. sanguineus* and *Hyalomma excavatum* were resistant to infection with both *H. bacteriophora* HP88 and *S. carpocapsae* DT. They found that *H. bacteriophora* HP88 caused 1.7% and 6% mortality in engorged larvae of *R. sanguineus* and *Hy. excavatum*, respectively. *Steinernema carpocapsae* DT caused 2.5% mortality in *R. sanguineus* larvae and failed to infect *Hy. excavatum* larvae. The resistance of larvae to EPNs may be attributed to the limited size of their natural openings which reduce the ability of IJs to penetrate their bodies. Additionally, the engorged larvae are capsulated and harden rapidly to molt to the next stage (nymph). The capsulated larvae might prevent IJs from penetrating through their cuticle. In this study, the effect of EPNs extended to nymphs molted from engorged larvae and the mortality rate was much higher, up to 38%. In contrast, in the experiment conducted by Samish *et al.* (1999), the mortality of unfed nymphs of the two tick species was lower than 10%. The high mortality of unfed nymphs in our study may be due to lower activity of nymphs after molting from engorged

Table 4 LC₅₀ and LC₉₅ values of *Rhipicephalus sanguineus* engorged females exposed to entomopathogenic nematodes *Heterorhabditis bacteriophora* HP88 and *Steinernema* sp. SII on the 5th day post-exposure.

Nematode species	LC ₅₀ (IJs/ml)			LC ₉₅ (IJs/ml)		
	Value	Confidence limits		Value	Confidence limits	
		Lower	Upper		Lower	Upper
<i>Heterorhabditis bacteriophora</i> HP88	76	19	151	5421	3029	16923
<i>Steinernema</i> sp. SII	885	766	1020	5700	4303	8302

larvae and the fact that their cuticles were not completely hardened at exposure, giving a chance for nematode penetration.

In our study, it was found that the engorged nymphal stage of *R. sanguineus* was more susceptible to EPN infections than the engorged larval stage; as well, the nematode species *Steinernema* sp. SII was more pathogenic than *H. bacteriophora* HP88. Both species caused mortality in engorged nymphs reaching 78% and 36%, respectively. These findings are in line with that recorded by Samish *et al.* (1999). They found that *H. bacteriophora* HP88 caused 30% and 15% mortality in engorged nymphs of *R. sanguineus* and *Hy. excavatum*, respectively. These values were slightly higher for *S. carpocapsae* DT, with 50% and 20% mortality in *R. sanguineus* and *Hy. excavatum* nymphs, respectively. Furthermore, in our study, the nematode *Steinernema* sp. SII achieved higher mortality (44% to 62%) in adults that emerged from engorged nymphs of *R. sanguineus* than the nematode species *H. bacteriophora* HP88 (32% - 50%). In agreement with this finding, Butt *et al.* (2016) recorded 30% and 40% mortality in unfed males and females of *Hy. excavatum* exposed separately to *Steinernema* sp. SII. The higher pathogenicity in engorged nymphs and emerged adults exposed to *Steinernema* sp. SII might be due to the greater ability of these nematodes to search and find ticks compared to *H. bacteriophora* HP88.

The effect we measured extended to days 13, 18, and 24 post-treatment. This is a considerably long time for an effective control method against active life stages and indicates that EPNs remain alive in the soil and can still infect the molted life stages. We could expect the EPNs to have similar behavior in the field, if the environmental conditions are suitable, especially moisture. Their impact in the field could also be improved by the availability of insect cadavers, such as from the order Lepidoptera, which as competent hosts could amplify the presence of EPNs in the environment. As the brown dog tick *R. sanguineus* spends more than 95% of its life cycle off the host hiding in cracks and crevices during its non-parasitic periods (Needham and Teel 1991; Dantas-Torres 2010), the application of EPNs as biocontrol agents would need to target these areas (Samish *et al.* 2008; Monteiro *et al.* 2014).

The calculated LC values of engorged females exposed to the nematode species *H. bacteriophora* HP88 and *Steinernema* sp. SII suggested that *H. bacteriophora* HP88 is more pathogenic than *Steinernema* sp. SII. This finding might be attributed to the presence of the anterior tooth of the *H. bacteriophora* HP88 that allows direct penetration through soft host cuticle into the body cavity (Hill 1998), in addition to IJs penetrating through natural openings. In agreement with this study, many previous studies found a pathogenic effect of different species of EPNs belonging to the genera *Heterorhabditis* and *Steinernema* on the females of different tick species. *Heterorhabditis bacteriophora* HP88 and LPP7 remained active against engorged females of *R. microplus* in the soil for 65 days after the application of EPN-infected *G. mellonella* cadavers (Monteiro *et al.* 2020). *Steinernema riobrave* killed 89% of the *R. sanguineus* fully engorged females, whereas *S. feltiae* killed 71% when using the petri dish technique which contained 15 g of sifted sand moistened with 5,000 IJs of nematodes (Kocan *et al.* 1998). Engorged females of *R. microplus* exposed to different EPN species revealed minimum LC₅₀ values of 3,180 (2,958-3,419) and 491 (462-522) nematodes per dish for *S. riobrave* and *H. floridensis*, respectively (Singh *et al.* 2019). Five isolates of heterorhabditidae and 2 isolates of steinernematidae were tested on the fully engorged females of *B. annulatus* ticks. The calculated LC₅₀ of *Heterorhabditis indica* RM1, *Heterorhabditis* sp. ISK1, *H. bacteriophora* TWF, *Steinernema carpocapsae* All and *S. riobrave* were 52, 63, 636, 2375, and 5700 IJs/mL, respectively (El-Sadawy and Abdel-Shafy 2007).

The normal hosts of EPNs are soil-inhabiting insects, especially belonging to the orders Lepidoptera and Coleoptera. EPNs complete their life cycle in these normal insect hosts and have the ability to develop for many generations. In the order Acarina (e.g. ticks), IJs have the ability to infect ticks, but with no development into the next generation (Lacey and Georgis, 2012; Singh *et al.* 2022). The use of EPNs for the biocontrol of ticks could have numerous benefits such as: a) zero toxicity to vertebrates, b) infection of a wide host range of soil insect pests that maintain and increase their populations in the field, c) easy movement in sandy soil

with a water film of sufficient thickness, d) an effective insecticidal activity at temperatures that range between 18–28 °C, e) a wide tolerance range of soil pH levels (4 to 8), and f) a weak impact on non-target arthropods including non-pest species of Coleoptera and Diptera that include predators and parasitoids (Shapiro *et al.* 1996; Campos-Herrera, 2015). On the other hand, there are some biotic (e.g., host availability, plant metabolites, and natural enemies) and abiotic (e.g., low moisture, temperature extremes, UV light, dehydration, and soil type) factors which are considered limitations for application of EPNs in the fields (Campos-Herrera, 2015). In addition, nitrogenous compounds, such as fresh animal manure and urea, which may be readily present in environments used by ticks, may reduce the survival of EPNs (Shapiro *et al.* 1996). More field experiments are thus needed to ensure the efficacy of EPNs on the target arthropods, the safety of EPNs for non-target arthropods and, to avoid imbalances in the soil ecosystem food webs. Moreover, studies that examine the application of EPNs in the cracks and crevices of buildings that animals are kept in are necessary to examine the effectiveness of this method for *R. sanguineus* ticks.

Conclusion

The entomopathogenic nematodes *H. bacteriophora* HP88 and *Steinernema* sp. SII revealed pathogenicity against the non-parasitic life stages of *R. sanguineus*. This pathogenicity was high on engorged nymphs and engorged adult females and low on engorged larvae. *Steinernema* sp. SII was more effective on engorged nymphs than *H. bacteriophora* HP88, while the latter species exhibited the highest effect on engorged females. A concentration of 2000 IJs/mL of both EPN species is the recommended dose that could be used in controlling stages of *R. sanguineus* (off-host).

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Author contribution

All authors shared in the design of the study. HSMA, HFA, SHMH and SA established *R. sanguineus* colony. HAE prepared entomopathogenic nematode concentrations. HSMA, HFA, SHMH, HAE and SA participated in tick exposure to nematodes and followed ticks after treatment. HSMA, SA analyzed the data and wrote the draft of the manuscript. All authors revised the final version of the manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

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