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Action of *Heterorhabditis indica* (Rhabditida: Heterorhabditidae) strain LPP1 on the reproductive biology of engorged females of *Rhipicephalus microplus* (Acari: Ixodidae)

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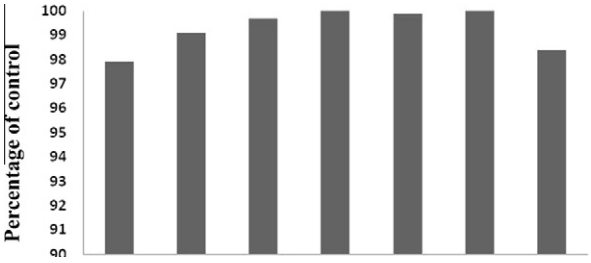
HIGHLIGHTS

- The action of the nematodes resulted in a significant reduction of all parameters.
- The efficacy of treatment in all groups was above 97%.
- *Heterorhabditis indica* LPP1 is the one of most virulent species for females of *Rhipicephalus microplus*.
- *H. indica* LPP1 is a promising agent for biological control of *R. microplus*.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of this study was to evaluate the effect of different concentrations of the entomopathogenic nematode (EPN) *Heterorhabditis indica*, LPP1, on the reproductive biology of engorged females of *Rhipicephalus microplus*. For this purpose, 240 females were divided into eight groups with statistically similar weights ($p > 0.05$). Each group was divided into six subgroups with five females distributed in Petri dishes and exposed to concentrations of 0, 375, 750, 1500, 3000, 6000, 12,000 and 24,000 EPNs/dish. The following biological parameters were evaluated: egg mass weight (mg), egg production index (%EPI), hatching percentage (%) and percentage of control. The action of the nematodes resulted in a significant reduction ($p < 0.05$) in egg mass weight, hatching percentage and EPI in all treatments groups. The percentage of control in all groups was above 97%, reaching 100% at concentrations of 3000 and 12,000 EPNs/female. Comparison of these results with those reported in other articles evaluating the in vitro pathogenicity of different EPN species against *R. microplus* shows that under laboratory conditions *H. indica* LPP1 is the one of most virulent species.

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1. Introduction

Rhipicephalus (*Boophilus*) *microplus* (Canestrini, 1888) (Acari: Ixodidae) is a common ectoparasite of cattle and is responsible for significant economic losses to beef cattle herders and dairy farmers in many countries (Martins et al., 2006; Furlong et al., 2007). It is estimated that in Brazil the economic losses generated by this tick amount to over two billion dollars annually (Grisi et al., 2002). The primary means to control this ectoparasite is through the application of acaricides, but their widespread use without following the proper technical criteria has resulted in the selection of ticks resistant to almost all chemical compounds available currently (Furlong et al., 2007; Labruna, 2008; Klafke, 2008). Additionally, the improper and indiscriminate use of acaricides can cause poisoning of animals and humans, due to chemical residues left in the environment and food (Kaaya et al., 2000; Samish, 2000; Samish and Glazer, 2001).

New alternatives to combat this arthropod have been studied with a view to reducing dependence on the use of acaricides. Studies of the biological control of *R. microplus* using EPNs in the laboratory have shown that EPNs are pathogenic to ticks, but the virulence varies according to species and strain of the nematode used (Vasconcelos et al., 2004; Freitas-Ribeiro et al., 2005; Monteiro et al., 2010a,b, 2012).

Another important aspect that should be taken into account in use of nematodes to control pests is the utilization of native strains, which are more adapted to the local climate and fauna, since, exotic species can be harmful to the environment where they are introduced (Dolinski and Moino-Jr, 2006). Therefore, the Embrapa Dairy Cattle Research Unit (Embrapa Gado de Leite) is conducting a research program to investigate interactions between ticks and EPNs to yield information on new ways to control cattle ticks. In this context, this study aimed to evaluate the effect of different concentrations of infective juveniles (IJs) of *Heterorhabditis indica* Poinar, Karanukar and David, 1992 strain LPP1 (from the city of Monte Negro, Rondônia, Brazil) on the reproductive biology of engorged females of *R. microplus*.

2. Material and methods

The study was carried out in the Parasitology Laboratory of the Embrapa Dairy Cattle Research Unit, located in Juiz de Fora, Minas Gerais, Brazil. Were used engorged females of a strain of *R. microplus* (Porto Alegre strain) maintained through artificial infestation on cattle at the Embrapa Dairy Cattle Experimental Field Station in the municipality of Coronel Pacheco, Minas Gerais, Brazil. The nematodes of the species *H. indica* LPP1 used in this study were donated by laboratory of nematology of North Fluminense State University. These nematodes were bred according to Lindegren et al. (1993); Kaya and Stock (1997).

The experiment was based on the method used by Vasconcelos et al. (2004). The engorged females were divided into eight groups of 30 ticks with statistically similar weights ($p > 0.05$) (Table 1). Each group was divided into six subgroups with five females, identified with nontoxic paint for individual monitoring (each female = an experimental unit), and distributed in Petri dishes (6 cm in diameter) containing 15 g of sterilized sand as substrate.

Each subgroup was sprayed with 4 ml of aqueous solution containing EPNs at concentrations of 375, 750, 1500, 3000, 6000, 12,000 and 24,000 IJs per Petri dish. The control consisted of 4 ml of distilled water free of nematodes. The groups were kept in a climate-controlled chamber at 27 ± 1 °C and RH > 80 ± 10% for a period of 48 h. The females were observed daily to check for mortality and egg-laying. The egg masses were placed individually in labeled 10-ml adapted syringes, and kept in the climate-controlled chamber under the same temperature and relative humidity conditions mentioned above.

After 48 h, the females still alive in each treatment were removed from the test dish and individually placed in plastic jars with lids (3 cm in diameter by 2 cm the height) and kept in the same chamber at 27 ± 1 °C and RH > 80%, for continued monitoring of reproductive biology. The following parameters were evaluated: egg mass weight (mg), hatching percentage (%) and the values were used to calculate the egg production index and efficacy of treatments.

The egg production index (%EPI) was obtained according to the equation proposed by Bennett (1974). EPI = egg mass weight × 100/female weight before oviposition. The EPI evaluates how much of the ingested blood per female was converted into eggs. The percentage of control of treatments, through offspring inhibition was obtained according to Drummond et al. (1973). First we calculated the index of estimated reproduction (ER): (egg mass weight/female weight before oviposition) × hatching percentage × 20,000. Next, was calculated the percentage of control: (ER of group control−ER of treated group)/ER of control group × 100.

The statistical analysis was performed using the software Biostat version 5.0. The percentage values were transformed into $\sqrt{}$arcsine x. The median values of each treatment were analyzed by ANOVA and the Tukey test ($p < 0.05$). In the case of nonparametric distributions, the values were compared through the nonparametric tests of Kruskal–Wallis and Student–Newman–Keuls ($p < 0.05$).

3. Results

H. indica LPP1 caused a reduction of egg mass weight, with significant differences ($p < 0.05$) between the treated groups (where the weight ranged from 9.83 to 0.13 mg) and the control group (114 mg). Among treatments, the groups treated with 375, 750 and 24,000 EPNs/dish were statistically similar ($p > 0.05$) to each

Table 1
Mean female weight before oviposition (mg), egg mass weight (mg), egg production index and hatching percentage (%) of *Rhipicephalus (Boophilus) microplus* treated with different concentrations of infective juveniles of *Heterorhabditis indica* LPP1, under laboratory conditions (27 ± 1 °C and RH > 80 ± 10%) and percentage of control.

Concentration of nematodes per petri dish	Female weight before oviposition (mg)	Egg mass weight (mg)	Hatching percentage (%)	Egg production index (% EPI)	Percentage of control (%)
0	230.9^{a} ± 43.2 (29)	114.5^{a} ± 31.1 (29)	91.2^{a} ± 14.7 (29)	49.24^{a} ± 9.09 (29)	
375	231.6^{a} ± 44.4 (29)	9.8^{b} ± 9.8 (29)	22.3^{b} ± 27.2 (28)	4.03^{b} ± 3.52 (29)	97.9
750	232.4^{a} ± 36.0 (29)	6.8^{b} ± 7.3 (29)	12.5^{b} ± 21.3 (26)	2.59^{b} ± 2.74 (29)	99.2
1500	232.5^{a} ± 36.2 (30)	2.2^{c} ± 4.4 (30)	5.0^{b} ± 10.8 (10)	0.88^{c} ± 1.85 (30)	99.7
3000	231.7^{a} ± 45.1 (30)	0.1^{c} ± 0.3 (30)	0.0*±0.0 (04)	0.06^{c} ± 0.17 (30)	100.0
6000	231.6^{a} ± 45.6 (30)	0.3^{c} ± 1.0 (30)	2.0^{b} ± 4.5 (05)	0.14^{c} ± 0.41 (30)	99.9
12,000	232.0^{a} ± 37.4 (30)	0.1^{c} ± 0.2 (30)	0.0*±0.0 (01)	0.18^{c} ± 0.10 (30)	100.0
24,000	232.7^{a} ± 36.5 (29)	7.0^{b} ± 25.2 (29)	23.0^{b} ± 40.1 (06)	2.72^{b} ± 9.87 (29)	98.4

(n): Sample size. Means followed by equal letters in the same column do not differ statistically at 5% significance.
* Statistical test not performed due to the small sample size.

other and differed significantly ($p < 0.05$) from the other treatments (1500, 3000, 6000 and 12,000 EPNs/dish), where the reduction of egg mass was more pronounced (Table 1).

Regarding the egg production index (EPI), were observed to treatments with 375, 750, 1500, 3000, 6000, 12,000 and 24,000 values between of 4.03%, 2.59%, 0.88%, 0.06%, 0.14%, 0.18% and 2.72% differing significantly ($p < 0.05$) from control (49.2%). It is noteworthy that significant differences were found ($p < 0.05$) between groups treated with 1500, 3000, 6000 and 12,000 EPNs/dish and the other treatments (350, 750 and 24,000 EPNs/dish) (Table 1).

Besides decreasing the egg mass of females in the treated groups, the exposure to the EPNs also led the production of infertile eggs, since the hatching percentage of all treated groups ranged from 23.0% to 0.0%, with significant differences ($p < 0.05$) compared to the control group, where the hatching rate was 91.2% (Table 1).

The lower percentage of control (97.9%) was obtained for the concentration of 375 EPNS/dish. The following concentrations were observed values above 99% reaching 100% at concentrations of 3000 and 12,000 EPNs/dish. At the highest concentration was observed a slight decrease in percentage of control which was 98.4% (Table 1).

4. Discussion

Different nematode species have shown varying pathogenicity levels to various tick species (Kaaya et al., 2000; Samish, 2000). Although Mauelon et al. (1993) tested 17 EPN strains and concluded that *R. microplus* was not susceptible to infections by any of these EPN species. However, more recent studies have demonstrated that certain EPNs are promising agents to control of the cattle tick (Vasconcelos et al., 2004; Freitas-Ribeiro et al., 2005; Monteiro et al., 2010a,b). The results of this study corroborate those findings.

H. indica LPP1 caused a reduction in the average egg mass size on a per/female basis. These results show that 48 h of exposure to the IJs was sufficient for the nematodes to penetrate and cause changes in this parameter, often killing the female before the onset of oviposition. These data differ from those reported by Vasconcelos et al. (2004), who used different concentrations of *Heterorhabditis bacteriophora* (Poinar, 1976) CCA and *Steinernema glaseri* (Steiner, 1929) Santa Rosa and reported that only concentrations of 5000 and 25,000 EPNs per dish of the *S. glaseri* caused a significant reduction in the egg mass. In contrast to the present study Monteiro et al. (2010b) evaluating the potential of *Heterorhabditis amazonensis* Andalo, Nguyen, and Moino-Jr, 2006 to control *R. microplus*, reported that only the highest concentration (6000 EPNs per dish) did not cause significant reductions in the egg mass weight. The results observed in the present study are similar to those found by Monteiro et al. (2010a) who reported that all the concentration of *H. bacteriophora* HP88 caused significant declines in the egg mass weight.

With respect to the EPI, the values obtained in the present study are similar to those found in experiments with *Steinernema carpocapsae* (Weiser, 1955) strains ALL and Santa Rosa (Freitas-Ribeiro et al., 2005) and *H. bacteriophora* HP88 (Monteiro et al., 2010a), where the values found in all the treatments were significantly lower than those for the control group. However, Vasconcelos et al. (2004) concluded that none of the concentrations of *H. bacteriophora* CCA and *S. glaseri* Santa Rosa studied led to a reduction of the EPI. The egg production index measures to what extent the blood ingested by the female is converted into eggs (Bennett, 1974). The significantly lower values indicates that the nematode tested interfered in the process of converting nutrients into eggs, causing the engorged females to die before the start of oviposition.

The utilization of the nematode *H. indica* LPP1 not only caused a reduction in the egg mass weight, it also led to the production of infertile eggs, since the hatching percentage declined significantly in all the treated groups. A similar result was obtained for *S. carpocapsae* (Weiser, 1955) strains ALL and Santa Rosa (Freitas-Ribeiro et al., 2005) and *H. bacteriophora* HP88 (Monteiro et al., 2010a) . However, this effect was not observed in relation to *H. bacteriophora* CCA and *S. glaseri* Santa Rosa (Vasconcelos et al., 2004) and *H. amazonensis* RSC-5 (Monteiro et al., 2010b), in which cases the hatching success rate of the treated groups were statistically similar to those of the control. A possible explanation for this fact is the possible deleterious action of the nematodes in the oviposition and/or embryonation process, by interfering in the steps of forming oocytes, fertilization, water absorption by the cuticle and waxing of the eggs by Gené's organ. For a better understanding of these results, it will be necessary to perform histological studies and evaluate the lipid and protein profile of the eggs and organs of female ticks infected by EPNs.

The percentage of control was greater than 97% for all the concentrations tested, reaching 100% at concentrations of 3000 and 12,000 EPNs/dish, exceeding the results observed with the use of *H. bacteriophora* CCA (Vasconcelos et al., 2004) and *H. amazonesis* RSC-5 (Monteiro et al., 2010b), where the greatest percentage of control were 80% and 67%, at concentrations of 1500 and 1500 EPNs/dish, respectively. Vasconcelos et al. (2004) used *S. glaseri*, related percentage of control above 97% only with the concentration of 25,000 EPNs/dish. Similar results were obtained by Monteiro et al. (2010a), who using concentrations of 375 at 6000 EPNs/dish of *H. bacteriophora* HP88, observed that all the concentrations resulted in percentage of control greater than 90%, however, values above 97% were only obtained in the highest concentration (6000 EPNS/dish) evidencing that results of the present study were even better. At the highest concentration (24,000 EPNS/dish) occurred a slight decrease in percentage of control, being observed value lower than of the previous concentrations. Vasconcelos et al. (2004), studying the effect of *H. bacteriophora* CCA observed that the most efficient control was achieved by a concentration of 1500 EPNs/dish and that at concentrations of 2500, 5000, and 25,000 EPNs/dish the efficacy declined fact also reported by Monteiro et al. (2010b) used *H. amazonensis* against *R. microplus*. This fact was attributed to intraspecific competition due to the high number of EPNs in these concentrations.

According to the results of the current study and others previously reported concerning the pathogenicity of different EPNs against the cattle tick, *H. indica* LPP1 represents one of the most virulent species of these entomopathogenic organisms. Under laboratory conditions, this nematode caused deleterious effects on the reproductive biology of *R. microplus* even when the lowest EPNs concentration was used, reducing the number and viability of the eggs from infected engorged females. Consequently, the potential of *H. indica* LPP1 to controlling the cattle tick must be investigated additionally under field conditions.

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