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Potential of entomopathogenic nematodes of the genus *Heterorhabditis* for the control of *Stomoxys calcitrans* (Diptera: Muscidae)

Potencial de nematoides entomopatogênicos do gênero *Heterorhabditis* para o controle de *Stomoxys calcitrans* (Diptera: Muscidae)

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Abstract

The present study verified the pathogenic potential of entomopathogenic nematodes (EPNs) of the genus *Heterorhabditis* (*Heterorhabditis bacteriophora,* isolate HP88 and *Heterorhabditis baujardi* isolate LPP7) to immature stages of *Stomoxys calcitrans* in the laboratory. All EPN concentrations of the *H. bacteriophora* HP88 strain caused mean larval mortality greater than 90% after four days. Higher concentrations of the *H. baujardi* LPP7 isolate (≥50 EPNs/larva) eliminated more than 70% of larvae after six days with the concentration 200 EPNs/larva reaching mortality levels of 93.3%. The larval mortality at all concentrations of EPNs (25, 50, 100, 150, and 200 EPNs/larva) for both strains was significant ($p<0.05$) when compared to the respective control groups. Concentrations of *H. bacteriophora* HP88 yielded an LC50 of 0.36 EPN/larva and LC90 of 29.1; while *H. baujardi* LPP7 yielded an LC50 of 39.85 and LC90 of 239.18. *H. bacteriophora* HP88 provided greater inhibition of the emergence of adults when compared to the response obtained with *H. baujardi* LPP7. EPNs did not cause considerable mortality when applied directly to pupae. The set of observed results suggests that the EPNs of the genus *Heterorhabditis,* isolates HP88 and LPP7, are a promising alternative in the control of the stable fly.

Keywords: Stable fly, diptera, biological control, *Heterorhabditis bacteriophora* HP88, *Heterorhabditis baujardi* LPP7.

Resumo

O presente estudo verificou o potencial patogênico de nematoides (NEP) do gênero *Heterorhabditis* (*H. bacteriophora* - isolado HP88 e *H. baujardi* - isolado LPP7) para estágios imaturos de *Stomoxys calcitrans* em laboratório. Todas as concentrações de NEPs da cepa HP88 causaram mortalidade larval média maior que 90% após quatro dias. Concentrações mais elevadas da cepa LPP7 (≥50 NEPs/larva) eliminaram mais de 70% após seis dias, com a concentração 200 NEPs/larva atingindo níveis de 93,3%. A mortalidade larval em todas as concentrações de NEPs (25, 50, 100, 150, and 200 NEPs/larva) para ambas as cepas foi significativa ($p<0,05$), quando comparadas aos respectivos grupos controle. Concentrações de *H. bacteriophora* HP88 obtiveram LC50 de 0,36 NEP/larva e LC90 de 29,1; enquanto *H. baujardi* LPP7 obteve LC50 de 39,85 e LC90 de 239,18. O isolado *H. bacteriophora* HP88 propiciou maior inibição da emergência de adultos, quando comparado à resposta obtida com *H. baujardi* LPP7. NEPs não provocaram mortalidade considerável quando aplicados diretamente sobre pupas. O conjunto de resultados observados indica os nematoides entomopatogênicos do gênero *Heterorhabditis* (estirpes HP88 e LPP7) como uma alternativa promissora no controle da mosca dos estábulos.

Palavras-chave: Mosca dos estábulos, diptera, controle biológico, *Heterorhabditis bacteriophora* HP88, *Heterorhabditis baujardi* LPP7.

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Introduction

Stomoxys calcitrans, a hematophagous Diptera known as "stable fly", performs its growth in organic matter - development of the immature stages occurs mainly in animal feces associated with decomposing vegetable matter (COOK et al., 1999). After hatching, the larvae move deeper into organic matter for feeding and protection, requiring two weeks to reach the pupal stage (MELLO, 1989). According to Taylor et al. (2012), this fly is present in several regions of the world, including temperate climates, which, in certain circumstances, offer conditions for its development during only some months of the year, yet the fly can still cause considerable damages.

Taylor et al. (2012) indicated that annual losses in the United States reached 2,221 billion dollars. In Brazil, Grisi et al. (2014) estimated losses at US$ 335.5 million. Evaluating the effect of *S. calcitrans* parasitism on cattle, Campbell et al. (1987) found that 35 flies per front leg were sufficient to cause losses of 12% in feed conversion and 20% in weight gain. The hematophagous habit and interrupted feeding, as well as the painful biting of the stable fly (BITTENCOURT, 2012), lead to defensive movements of the host to fend off the flies, which then land on another susceptible animal, thus being able to transfer pathogen-infected blood remaining in its mouthparts, facilitating the transmission of several pathogenic microorganisms (BALDACCHINO et al., 2013).

One class of agents with potential for biological control of the stable fly is the entomopathogenic nematodes ("EPNs"), which are obligatory parasites of insects and have a wide geographical distribution. EPNs of the genus *Steinernema* and *Heterorhabditis* are associated with symbiotic bacteria of the genus *Xenorhabdus* and *Photorhabdus*, respectively (BOEMARE et al., 1993). They search for and locate their hosts by products of excretion, CO_2 levels and temperature gradients. When a host is located, they penetrate through natural openings (mouth, anus and spiracles) or actively penetrate through the cuticle, as in the case of *Heterorhabditis* sp. Inside the host, they migrate to the hemocoel (KAYA & GAUGLER, 1993) where their symbiotic bacteria release toxins that kill the insect (HAZIR et al., 2003). The infective juveniles (IJ) then ingest decomposed tissues and the bacteria inside the host as they develop into adults. These nematodes can complete two to three generations inside the insect until nutrients are depleted, at which point the IJ (= J3) retain the bacteria and leave the dead insect to seek a new host in the environment (DOLINSKI et al., 2006).

EPNs have several characteristics making them desirable as control agents: they are highly virulent, easily cultivated *in vitro*, possess a large array of hosts, are highly specific to arthropods, are mobile and have high reproductive potential (KAYA & GAUGLER, 1993). Currently, these nematodes are already used commercially for the control of agricultural pests in different regions of the planet (GEORGIS & MANWEILER, 1994).

In relation to parasites of veterinary importance, most studies have used EPNs for control of ticks (SAMISH et al., 2008; MONTEIRO et al., 2010; SILVA et al., 2012; MONTEIRO, 2014) with few existing studies on the potential of these agents for control of flies. Notable exceptions include studies by Geden et al. (1986) and Taylor et al. (1998) with *Musca domestica* (Diptera: Muscidae) and Mahmoud et al. (2007) and Pierce (2012) with *S. calcitrans*.

The objective of the present study was to evaluate the pathogenic potential of the nematodes *Heterorhabditis bacteriophora*, and *Heterorhabditis baujardi* to immature *Stomoxys calcitrans* under laboratory conditions.

Materials and Methods

Location of experiment

The bioassays were conducted at the Research Laboratory of Hematophagous Diptera located at the Station for Parasitological Research W. O. Neitz (EPPWON), Veterinary Institute, Federal Rural University of Rio de Janeiro (UFRRJ), Brazil.

Obtaining larvae of S. calcitrans

The capture of adult *S. calcitrans* was carried out in cattle at the Veterinary Hospital of UFRRJ using an entomological net. Flies were stored in plastic transport cages (15x15x20 cm) and identified in a laboratory according to Furman & Catts (1982). For the rearing of *S. calcitrans* under laboratory conditions, cages of plastic material measuring 60x40x50 cm were used (MORAES, 2007). Adult rearing cages and larval development media were kept in a climatized chamber (B.O.D.) at 27±1°C and 70-80% relative humidity (RH). Bovine blood was transferred to a beaker, heated in a water bath (approximately 37°C) to simulate the body temperature of the parasitized animal (MELLO, 1989), and supplied to the flies on a gauze pad twice a day. For the maintenance of larvae, modified Christmas larval rearing medium (CHRISTMAS, 1970) composed of sugar cane (66 g), wheat bran (25 g), meat meal (8 g), sodium bicarbonate (1 g) and distilled water (127 mL), was used.

Obtaining and maintaining nematodes

The nematodes *H. bacteriophora* strain HP88, and *H. baujardi* strain LPP7, were obtained from the Parasitology Laboratory at EMBRAPA Dairy Cattle, Juiz de Fora, Minas Gerais, Brazil. The nematodes were maintained in cell culture flasks (40 mL capacity) containing 20 mL of aqueous suspension with EPNs and were quantified by counting 10 aliquots of 10 µL. In each aliquot (10 µL), the viable larvae were counted, and then the mean number of infective juveniles (IJ) was calculated per sample. From this mean, the suspensions were adjusted, by adding or removing distilled water from the bottles (MONTEIRO, 2014).

Exposure of larvae and pupae of S. calcitrans to nematodes

To perform the larval susceptibility test for each nematode species, the experimental unit consisted of five eight-day-old larvae placed in a Petri dish (9 cm in diameter) lined with two sheets of filter paper in the lower part. To each plate, 2 mL of aqueous suspension of nematodes at the concentration of either 125, 250, 500, 750 or 1000 EPNs/Petri dish (each concentration = one treatment) were added. Thus, the concentrations per larva

were 25, 50, 100, 150 and 200 EPNs. In the control groups, 2 mL of distilled water (nematode-free) were added to the plates with five *S. calcitrans* larvae. The Petri dishes were then sealed with PARAFILM® to prevent the escape of *S. calcitrans* larvae and stored in a climatized chamber at 27±1°C and 70-80% RH. Mortality of larvae was observed daily up to the 10th day after exposure. After this period, the observations were continued to evaluate the possible formation of pupae and adult emergence.

The evaluation of the effect of EPNs on pupae was conducted in a similar way to the bioassays involving larvae; the experimental unit consisted of five pupae with three days of formation, and EPN concentrations per pupa were 100, 150 and 200 EPNs. This segment of the study was performed only with EPNs of the species *H. bacteriophora* HP88, and the pupae were followed for up to 12 days to verify the emergence of adults. Six replicates were performed per treatment in both experiments.

White traps Dead *S. calcitrans* larvae were deposited in White traps (WHITE, 1927) according to each treatment for recovery of infective juveniles and confirmation of death due to infection by EPNs.

Statistical analysis

For statistical analysis, the software Biostat, version 5.0, was used. The values for mortality means in each treatment were analyzed by ANOVA and Tukey's test ($p < 0.05$). In the case of non-normal distribution, the parameters were compared by Kruskal-Wallis and Student-Newman-Keuls tests ($p < 0.05$). Determination of LC50 and LC90 was made using probit analysis on mortality data, and obtained together with 95% confidence upper and lower limits (FINNEY, 1952).

Results and Discussion

S. calcitrans larval viability

The usage of young *S. calcitrans* larvae in experiments carries two main risks: high mortality (control and treated groups) caused by the manipulation of larvae that are still fragile (MORAES, 2007) and mortality caused by lack of nutritional reserves in young larvae (L1 and L2). The latter is a particular risk in experiments where no development medium is provided in Petri dishes since *S. calcitrans* larvae require organic matter to feed and are dependent on this medium for protection from light and dryness. For this reason, in studies using freshly hatched *S. calcitrans* larvae, it is not recommended to adapt the methodology used by Senna-Nunes et al. (2002) with *Musca domestica*, a study which involved non-fed three-day-old larvae. In the bioassays described in the present study, when eight-day-old larvae were used, they were at a stage of development sufficient to withstand manipulation (withdrawal from development medium and passage to Petri dishes) and they possessed energy reserves sufficient to allow pupation, avoiding an artificial vulnerability to the action of entomopathogenic nematodes.

As performed by Pierce (2012), the current experiments were executed without the activation of photoperiods since, without the protection provided by the development medium, exposure of the larvae to light could become a stressor. Even without light sources, it was common to find larvae searching for shelter between or under the sheets of filter paper in Petri dishes as they exhibit positive geotropism. However, using a 14-hour photoperiod, Mahmoud et al. (2007) found low mortality in the control groups of their study. The same occurred in the present study and research performed for Pierce (2012), indicating that photoperiod does not affect larval viability under these conditions.

Bioassay with S. calcitrans larvae

The mortality of *S. calcitrans* larvae caused by two days of exposure to *H. bacteriophora* HP88 was 96.7% in the treatments with the three highest concentrations (100, 150 and 200 EPNs/larva) (Figure 1). After 10 days of exposure, there was a significant difference ($p < 0.05$) in mortality between all groups treated with EPNs and their respective controls in which high larval viability was observed (Table 1). However, no significant difference ($p > 0.05$) was observed between groups treated with different concentrations of EPNs of *H. bacteriophora* HP88. This result indicates that, under the conditions in which the study was carried out, this strain was highly virulent for *S. calcitrans* larvae even at relatively low concentrations. With regard to the *H. baujardi* LPP7, the 33.3% mortality in the group treated with 25 EPNs/larva differed significantly ($p < 0.05$) from treatments with concentrations of 150 and 200 EPNs/larva where mortality rates of 80.0% and 93.3% were found. No significant differences ($p > 0.05$) were observed among the other treated groups (50, 100, 150 and 200 EPNs/larva).

The emergence of adults in the control group for *H. bacteriophora* HP88 reached 80% of the total available samples representing

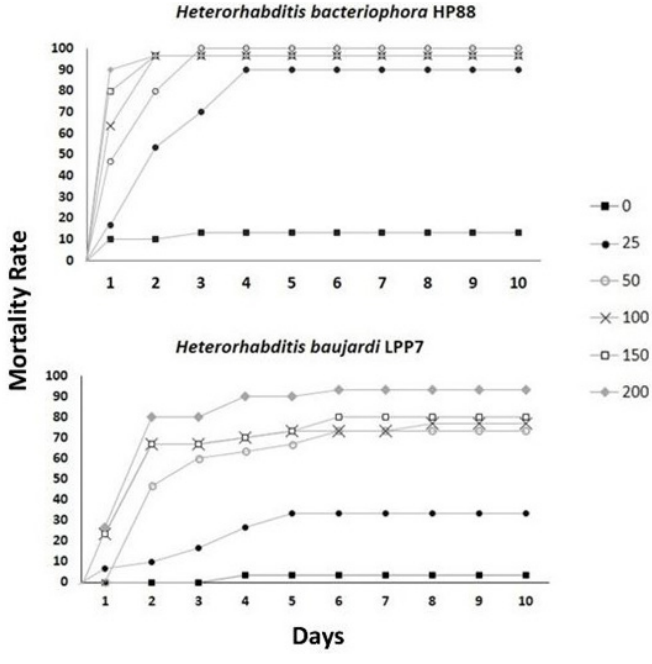

Figure 1. Cumulative mortality of third instar larvae of *Stomoxys calcitrans* exposed to different concentrations of *Heterorhabditis bacteriophora*, strain HP88, and *Heterorhabditis baujardi*, strain LPP7, under laboratory conditions (27 ± 1 ° C, 70-80% RH).

92.3% pupal viability. However, in the treated groups, total emergence was highly inhibited. Although the concentrations of 100, 150 and 200 EPN/larva did not achieve 100% larval mortality, pupae formed in these groups were not viable since there was no emergence of adults after two weeks of observation (Table 2). In the groups treated with *H. baujardi* LPP7, the emergence of adults decreased as the concentration of EPNs increased. The viability of the pupae formed in each group remained high with the exception of the two groups with the highest concentrations (150 and 200 EPNs/larva) (Table 2).

When comparing the results of larval mortality at the same concentrations of the two strains, *H. bacteriophora* HP88 had higher virulence and led to higher mortality ($p < 0.05$) than *H. baujardi* LPP7 except at the concentration of 200 EPNs/larvae in which similar results were observed for both species ($p > 0.05$) (Table 1). Thus, based on virulence tests, *H. bacteriophora* HP88 presents greater potential for control of *S. calcitrans*. This strain has also been identified as possessing the greatest potential for control of the tick *Rhipicephalus microplus* (MONTEIRO & PRATA, 2013). In *in vivo* experiments applying these nematodes in cadaver-insect

Table 1. Mean mortality of larvae of the third instar (eight days old) of *Stomoxys calcitrans* treated with different concentrations of *Heterorhabditis baujardi*, strain LPP7, or *Heterorhabditis bacteriophora*, strain HP88, under laboratory conditions (27±1°C and UR 70-80%) (Mean ± standard deviation).

EPN/larva concentrations	*Heterorhabditis bacteriophora* HP88	*Heterorhabditis baujardi* LPP7
0 (Control)	13.3^{a}±10.3	3.3^{a}±8.1
25	90.0^{bA}±16.7	33.3^{bB}±32.7
50	100.0^{bA}±0.0	73.3^{bcB}±20.7
100	96.7^{bA}±8.1	76.7^{bcB}±15.01
150	96.7^{bA}±8.1	80.0^{cB}±12.2
200	96.7^{bA}±8.1	93.3^{cA}±10.3

Means followed by different lowercase letters in the same column and by different capital letters on the same line differ statistically at the significance level of 5%.

Table 2. Mean percentages of pupal viability and emergence of *Stomoxys calcitrans* adults treated at the larval stage with different concentrations of *Heterorhabditis baujardi*, strain LPP7, or *Heterorhabditis bacteriophora*, strain HP88, under laboratory conditions (27±1°C and UR 70-80%).

Treatments	n	EPN/larva concentrations	Pupal viability	Adult emergence
H. baujardi LPP7	30	0 (Control)	93.1%	90%
	30	25	90%	60%
	30	50	89%	26.7%
	30	100	100%	23.3%
	30	150	59.8%	10%
	30	200	49.2%	3.3%
H. bacteriophora HP88	30	0 (Control)	92.3%	80%
	30	25	33.3%	3.3%
	30	50	0%	0%
	30	100	0%	0%
	30	150	0%	0%
	30	200	0%	0%

formulation, it was also found that *H. bacteriophora* HP88 showed better performance than *H. baujardi* LPP7 (MONTEIRO, 2014). However, such results are contrary to those of Dolinski et al. (2006) who found, using the two strains in tests under laboratory conditions, that *H. baujardi* LPP7 was more virulent for *Conotrachelus psidii* (Coleoptera: Curculionidae). These results show that the virulence of different species of EPNs varies according to the host, and it is always necessary to carry out specific tests to select the most virulent strains for a particular arthropod species.

In the groups of larvae treated with EPNs of *H. bacteriophora* HP88, there was no emergence of adults except in the group treated with 25 EPNs/larva (Table 2). This finding shows that, in the present study, infected larvae molted, but most of the pupae formed were not viable. Some authors (e.g., BEAVERS & CALKINS, 1984; TOLEDO et al., 2005) have reported that it is difficult for EPNs to penetrate through pupal spiracles; however, as the age of the larvae used in the current study was advanced enough for pupation, the molting occurred at the same time as the process of infection by EPNs.

Concentrations of *H. bacteriophora* HP88 yielded an LC50 of 0.36 EPN/larva (0.06-2.45) and LC90 of 29.1 (4.35-195.46) [slope: 0.674]; while *H. baujardi* LPP7 yielded an LC50 of 39.85 (23.45-67.74) and LC90 of 239.18 (140.73-406.5) [slope: 1.647].

Comparing results of this study with others investigating the use of EPNs for the biological control of *S. calcitrans* is a complicated task due to the limited number of such studies. The experiments performed by Mahmoud et al. (2007) involving different species and stages of flies (including *S. calcitrans*) under similar conditions to the present study show comparable results for third instar larvae. These authors, using the nematode *Steinernema feltiae* (Cross 33), observed high mortality rates of *S. calcitrans* larvae with concentrations close to those used in the present study (100% mortality at concentrations of 80 and 100 EPNs/larva after 72 hours). These results suggest that, in terms of pathogenicity on *S. calcitrans*, the species of *Heterorhabditis* used in this study offer potential for application as a control method for this dipteran, and require a low number of IJ to reach satisfactory levels of mortality in the laboratory.

Further supporting the use of *Heterorhabditis* spp. in controlling *S. calcitrans* are the tests performed by Pierce (2012) involving hay/manure. In these tests, both *Steinernema* spp. and *Heterorhabditis* spp. were employed and better results were obtained in treatments with the latter. The maximum mortality reached in the present study using *H. bacteriophora* HP88 and *H. baujardi* LPP7 was higher than that observed by Pierce (2012), which was 30.3%. Among all the concentrations used in the present study with the two strains of EPNs, the only one that presented values close to those found by Pierce (2012) was that of 25 EPNs/larva using *H. baujardi* LPP7; the other results were higher.

In their study evaluating the potential of EPNs for control of *M. domestica*, Geden et al. (1986) found that *Heterorhabditis heliothidis* (= *H. bacteriophora*) isolate North Carolina caused mortality close to that invoked by *Steinernema feltiae* (strain DD-136) on larvae and adults (27.6% and 34.8%, respectively, with a concentration of 100 EPNs/larva). Tracing a parallel between *S. calcitrans* and *M. domestica*, a relevant comparison due to their physiological similarities and phylogenetic proximity, it is notable that a much smaller number of infective juveniles of *H. bacteriophora* was required

in the present study to cause a high mortality of *S. calcitrans* larvae. Such a finding suggested either greater resistance of *M. domestica* larvae to the action of entomopathogenic nematodes or less virulence of the North Carolina strain when compared to the strain used in the present study.

In contrast, Taylor et al. (1998), who evaluated the pathogenicity of EPNs on larvae of *M. domestica* under experimental conditions similar to those used by Geden et al. (1986), observed that *Steinernema* spp. were more virulent than *Heterorhabditis* spp., even mentioning that none of the species of *Heterorhabditis* used presented potential for control of the domestic fly. From these results, we can infer that strains of the genus *Heterorhabditis* are not always highly virulent for all flies and that some strains of the genus *Steinernema* may present greater virulence than certain strains of *Heterorhabditis*.

Activity over S. calcitrans pupae

In the test with pupal exposure of *S. calcitrans* to EPNs, high pupal viability was observed in the different treatments regardless of the concentration used. Pupal viability values were 93.3%, 96.7% and 96.7% in the concentrations of 100, 150 and 200 EPNs/pupa, respectively, while in the control, pupal viability was 87.7%. No significant differences were observed between the control and treated groups ($p > 0.05$). These results are in accordance with the findings of several authors (e.g., BEAVERS & CALKINS, 1984; TOLEDO et al., 2005) regarding the limited ability of infective juveniles to penetrate pupal spiracles. It is possible that higher concentrations of these EPNs may promote greater mortality, but this aspect requires further investigation.

Retrieval of EPNs from White traps

The appearance of fly larvae in the groups treated with *Heterorhabditis* (Figure 2) is in accordance with Kaya & Gaugler's (1993) description. The dead larvae showed coloration tending to red/brown after their death and lost the natural glow of the integument, which is related to multiplication of the symbiotic bacteria, proliferation of EPNs and use of the insect as a nutritional source. Two weeks after assembling the White traps, a large number of infective juveniles was found in the water in Petri dishes.

Figure 2. Alteration of color and appearance of *Stomoxys calcitrans* larvae caused by infection by EPNs of the genus *Heterorhabditis*. (A) dead larvae after the attack from EPNs; (B) live larvae of the control group.

Conclusions

It was concluded that the *Heterorhabditis bacteriopho*ra HP88 and *Heterorhabditis baujardi* LPP7 showed pathogenicity to *S. calcitrans* larvae under laboratory conditions and were able to cause a high mortality; *H. bacteriophora* HP88 was the most virulent strain. However, pupae of *S. calcitrans* were resistant to infection by infective juveniles of these nematodes.

Despite this study being strictly under laboratory conditions, the finding of lower efficiency of *H. baujardi* LPP7, compared to *H. bacteriophora* HP88, in the control of *S. calcitrans* should not be a reason for discontinuation of bioassays involving that strain given that the efficacy levels demonstrated by both species are considerably high in terms of biological control, especially considering the concentrations used. The efficacy of *Heterorhabditis baujardi* LPP7 appears to be dose-dependent, and this isolate also has some positive characteristics for its applicability in Brazil: it is a native EPN collected in the Amazon region (DOLINSKI et al., 2008) and is adapted to the weather conditions of a tropical country.

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