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Native isolates and the effect of aviary litter on the pathogenicity and virulence of entomopathogenic nematodes for the control of the lesser mealworm, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae)

Isolados nativos e efeito da cama de aviário na patogenicidade e virulência de nematoides entomopatogênicos visando o controle do cascudinho de aviário, *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae)

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Highlights

Selection test with 14 native isolates from Brazil were undertaken.
The optimal concentration of NEPs were defined.
Effect of poultry litter on the virulence of nematodes was determined.

Abstract

Lesser mealworms cause great damage to poultry. Its control is difficult because of the insect's habit of living in the middle of the aviary bed. However, entomopathogenic nematodes (NEPs) can be an effective control of these insects, as they are indicated against pests that live or pass a phase in the soil. The objective of this study was to select native isolates of entomopathogenic nematodes and to evaluate the

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effect of poultry litter on its pathogenicity and virulence, with the aim of using these to control the lesser mealworm in poultry. Fourteen native isolates and two non-native isolates were used in the selection test, and the three most virulent isolates were used in the concentration test (10, 20, 40, and 50 infective juveniles (JIs) per cm²). Both experiments were performed in a completely randomized design, with the data from the selection test being submitted to the Scott-Knott mean test (P ≤ 0.05) and the concentration test to regression analysis. The effect of poultry litter (new and old) was evaluated on *Steinernema feltiae* (IBCB-n 47), *S. carpocapsae* (All), and *Heterorhabditis amazonensis* (UEL 08), and the test was conducted in 2 × 3 factorial (two types of litter and three isolates) design, and the data were subjected to Tukey's mean test (P ≤ 0.05). In the selection test with adult hosts, the three most virulent isolates were *H. amazonensis* (UEL 07), *H. amazonensis* (RSC 05)*, and S. carpocapsae* (all) with 76.5%, 73.5%, and 70% mortality, respectively. For larvae, the isolates *Heterorhabditis* sp. (NEPET 11), *S. feltiae* (IBCB-n 47), *H. amazonensis* (UEL 07), *Heterorhabditis* sp. (IBCB-n40)*, and H. amazonensis* (UEL 08) were the most virulent and differed statistically from the control. In the concentration test, the highest mortality in adults (98%) and larvae (98%) was observed with *S. feltiae* at concentrations of 30 JIs/cm² and 50 JIs/cm², respectively, and the native isolate evaluated (UEL 07) presented the worst performance. Regarding the effect of litter, it was observed that *S. feltiae* (IBCB-n 47) and *S. carpocapsae* (All) caused the highest mortalities, both in new litter (60.7% and 58.7%, respectively) and in old litter (80.0% and 74.7%, respectively), which were higher than that observed for the native isolate (UEL 08).
Key words: Biological control. *Heterorhabditis*. *Steinernema*.

Resumo

O cascudinho causa grande prejuízo para a avicultura. Seu controle é dificultado, devido ao hábito do inseto de viver em meio a cama de aviário. Por outro lado, os nematoides entomopatogênicos (NEPs) podem ser uma alternativa no controle destes insetos, pois são indicados contra pragas que vivem ou passam uma fase no solo. Desta forma objetivou-se selecionar isolados nativos de NEPs e avaliar o efeito da cama de aviário na sua patogenicidade e virulência, visando o controle do cascudinho de aviário. Foram utilizados 14 isolados nativos e dois isolados importados no teste de seleção e os três isolados mais virulentos foram utilizados no teste de concentrações (10, 20, 40, 50 juvenis infectantes (JIs)/cm²). Ambos experimentos foram realizados em delineamento inteiramente casualizado, sendo que os dados do teste de seleção foram submetidos a teste de média Scott-Knott (P ≤ 0,05) e o teste de concentrações à análise de regressão. O efeito da cama de aviário (nova e velha) foi avaliado sobre *Steinernema feltiae* (IBCB-n 47), *Steinernema carpocapse* (All), e *Heterorhabditis amazonensis* (UEL 08), e o ensaio foi conduzido em fatorial 2 x 3 (dois tipos de cama e três isolados), e os dados foram submetidos ao teste de médias Tukey (P ≤ 0,05). Observou-se no teste de seleção para adultos, que os três isolados mais virulentos foram *Heterorhabditis amazonensis* (UEL 07), *H. amazonensis* (RSC 05) e *S. carpocapsae* (All) com 76,5; 73,5; 70% mortalidade, respectivamente. Para larvas, os isolados *Heterorhabditis sp*. (NEPET 11), *S. feltiae* (IBCB-n 47), *H. amazonensis* (UEL 07), *Heterorhabditis* sp. (IBCB-n40) e *H. amazonensis* (UEL 08) foram os mais virulentos e diferiram estatisticamente. No teste de concentrações, a maior mortalidade em adultos (98%) e larvas (98%) foi observada para *S. feltiae* nas concentrações de 30 JIs/cm² e 50JIs/cm² respectivamente, e o isolado nativo avaliado (UEL 07) foi o que apresentou o pior desempenho. Com relação ao efeito da cama de aviário, observou-se que *S. feltiae* (IBCB-n 47) e *S. carpocapse* (All) causaram as maiores mortalidades, tanto em cama nova (60,7 e 58,7%)

quanto em cama velha (80 e 74,7%) respectivamente sendo também superiores ao desempenho do isolado nativo (UEL 08).

Palavras-chave: Controle biológico. *Heterorhabditis. Steinernema*.

Introduction

The "lesser mealworm", *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) is a cosmopolitan pest, originally from the African continent (Vaughan, Turner, & Ruszler, 1984), which uses broiler poultry litter as an economic environment at temperature, collect, shelter, and feed for their development (Japp, Bicho, & Silva, 2010). However, they destroy poultry litter and harbor several avian pathogens (Axtell & Arends, 1990), making them one of the main pest insects in poultry farming.

The lesser mealworm can be a vector and reservoir of pathogens such as those that cause the Newcastle disease, Gumboro disease, avian leukosis, and other diseases that cause immunosuppression in birds that can cause death (Despins & Axtell, 1995). In addition, birds are attracted to consuming both the adult stage and the larvae of this beetle, which decreases the daily rate of feed intake, resulting in considerable weight loss due to damages to the digestive tract caused by the rigidity of the elytron, leaving them even more vulnerable to infections (Japp et al., 2008).

The control of these pests is hampered by the presence of birds in the aviary. In addition, this insect inhabits cracks in the floor, and is often found concentrated below the feeders, drinking fountains, close to the supporting pillars of the buildings, and below the ground, which facilitates reinfection (L. F. A, Alves, Rohde, & Alves, 2005).

Chemical insecticides are commonly used for their control. However, when applied improperly, these can cause environmental problems, accumulate in poultry meat, facilitate the selection of resistant individuals, and can cause poisoning of poultry farmers (Rohde et al., 2006). Thus, it is necessary to look for non-chemical control alternatives that can be used on a commercial scale. Among the alternatives, biological control with entomopathogenic nematodes (NEPs) has been suggested (V. S. Alves, Neves, Alves, Moino, & Holz, 2012).

NEPs are obligate parasites, with important characteristics such as host specificity, ability to search for the host, virulence, and compatibility with other entomopathogens and chemical pesticides (Fuga, Fernandes, & Lopes, 2012; Guide, Alves, Fernandes, Ferreira, & Neves, 2016). In addition, these organisms show symbiotic relationships with bacteria, the main characteristic of the species of the two genera, *Heterorhabditis* and *Steinernema*, which are most commonly used in biological control. The majority of species of the genus *Heterorhabditis* are colonized by bacteria of the genus *Photorhabdus*, and species of the genus *Steinernema*, in general, are colonized by bacteria of the genus *Xenorhabdus* (Forst & Clarke, 2002). These bacteria when inoculated into the host's hemocele excrete lethal toxins, which cause the death of the host insect within 72 hours (Dolinski & Moino, 2006)

The species of the genera *Heterorhabditis* and *Steinernema* are able to move in the soil through water, a characteristic that should be highlighted in relation to other entomopathogens (Fuga et al., 2012). Lewis,

Campbell, Griffin, Kaya and Peters (2006) classified these nematodes into two categories based on their search strategy: "cruiser" (tracking) in which, the nematode moves in search of the host, and "ambusher" (ambush), in which, the nematode waits for the insect to ingest it when feeding to penetrate the insect's body. Infection of NEPs occurs mainly through penetration through natural openings (mouth, anus, and spiracles).

Geden, Axtell & Brooks (1985) found the efficiency of NEPs, especially *Steinernema glaseri*, *S. feltiae,* and *Heterorhabditis heliothidis*, to be high under laboratory conditions. In Brazil, some studies report the extraordinary efficiency of the species *S. carpocapsae* (L. F. A. Alves et al., 2005) and *S. arenarium* (V. S. Alves et al., 2012). However, in the field, it is important to first consider the use of native species (Dolinski & Moino, 2006), and avoid the risk of introducing exotics.

Thus, we aimed to select entomopathogenic nematodes that were native to Brazil, compare them with two non-native species, and evaluate the effect of poultry litter on the pathogenicity and virulence of these species for use in the control of avian mealworm.

Materials and Methods

Obtaining, multiplying, and storing of NEP isolates

The individuals used in the selection test belonged to the collection maintained at the Laboratory of Pathology and Microbial Control of Insects at the State University of Londrina (UEL), which were derived from nematodes provided by the Laboratory of Entomology and Microbial Control at the State University of Northern Paraná (Cornélio Procópio - PR) and the Instituto Biológico in Campinas, SP (Table 1).

For maintenance and use in the bioassays, they were multiplied in vivo, according to Molina and Lopes (2001), using the last stage of *Galleria mellonella* L. (Lepidoptera: Pyralidae).

Table 1
Isolates from entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* used in the selection test against larvae and adults of *Alphitobius diaperinus* under laboratory conditions

Isolates	Species	Origin
RSC 05	Heterorhabditis amazonensis	Benjamin Constant - AM - Brazil
NEPET11	*Heterorhabditis* sp.	Palmeira das Missões - RS - Brazil
ALHO-GL	*Heterorhabditis* sp.	Lavras - MG - Brazil
IBCB-n 40	*Heterorhabditis* sp.	Tabapuã - SP - Brazil
IBCB-n 05	*Heterorhabditis indica*	Itapetininga - SP - Brazil
IBCB-n 06	*Steinernema brazilense*	Porto Murtinho - MT - Brazil
IBCB-n 44	*Heterorhabditis* sp.	Santa Adélia - SP – Brazil
IBCB-n 46	*Heterorhabditis* sp.	Santo Antônio de Posse - SP - Brazil
JPM 4	*Heterorhabditis* sp.	Lavras - MG - Brazil
UEL 01	*Heterorhabditis amazonensis*	Londrina - PR - Brazil
UEL 07	*Heterorhabditis amazonensis*	Londrina - PR - Brazil
UEL 08	*Heterorhabditis amazonensis*	Londrina - PR - Brazil
PAM	*Steinernema rarum*	Aceguá - RS - Brazil
AM163	*Steinernema diaprepesi*	Sinop - MT - Brazil
All	*Steinernema carpocapsae*	Florida - USA
IBCB-n 47	*Steinernema feltiae*	Germany

Collection of lesser mealworm and obtaining chicken litter

The population of *A. diaperinus* and the seven month old litter (litter from the fifth batch; old) used in the tests were obtained from a poultry rearing house with soil floor, in the city of Londrina, Paraná (23°18'37''S; 51°09'46''W). As collections were made with gas, the bed surface was removed from under the base of feeders, where the insect population was more concentrated and was stored in a plastic container. This was kept at 25 ± 1 °C in the laboratory from June to October 2018. A 50 g portion of chicken feed was added to the container for feeding the insects.

The litter from the first batch, i.e., 45 days old litter (new) was collected from the poultry rearing sheds in the Fazenda Escola of the State University of Londrina (UEL), Londrina, PR. The bed was removed manually from the central part of the bay and placed in a 20 L plastic container. The storage was carried out in the same place and under conditions similar to that described above.

Selection of native isolates of entomopathogenic nematodes that affect Alphitobius diaperinus

For the selection test, 16 NEPs belonging to the genera *Heterorhabditis* and *Steinernema* from the entomopathogen bank were used (Table 1). The pathogenicity and virulence in the larvae and adults of *A. diaperinus* were determined following the

methodology of V. S. Alves et al. (2012) with changes in the larval test.

To test the effect of the NEPs on the adult beetles, each treatment consisted of four repetitions. Each repetition included a Petri dish, 9 cm in diameter, containing two filter papers at the base, and 15 insects. With the aid of a micropipette, concentrations of 100 juvenile infectious agents (JIs) per cm^2 with additional distilled water, totaling 2 mL of suspension per plate, were applied homogeneously on the paper. The insects were subsequently released into the Petri dish. The control followed the same procedure, but the suspension was replaced with 2 mL distilled water.

In the larval assay, each of the four replicates was represented by a 12-well cell culture plate. In each well (2.2 cm in diameter) two paper discs were placed followed by a single larva (in order to avoid cannibalism between them), totaling 12 larvae per repetition. JIs at a concentration of 100 JIs/cm^2 were applied to each well along with additional distilled water, totaling 0.18 mL per well. This was done immediately after the release of the larva in the water channel. In the control, only 0.18 mL of distilled water was applied to each well.

To standardize the tests, a concentration of 100 JIs/cm^2 was stipulated. The bioassays were conducted in a random isolated design and kept in an air-conditioned chamber at 25 ± 1 °C, UR70 ± 10%, without photoperiod, and without food. The evaluation took place after five days of exposure of insects to NEPs, after which the dead insects were dissected to confirm if the death was caused by NEP. Analysis of variance and the Scott-Knott media test ($P \leq 0.05$) were performed using the statistical program SISVAR, version 5.4 (Ferreira, 2011). The nematodes that caused the highest mortality were selected for the concentration tests.

Concentration tests

Six concentrations of the nematodes (0 (control), 10, 20, 30, 40, and 50 JIs/cm^2) were tested on the larvae and adults. Four replicates for each concentration for adults and for larvae were set up and the same methodology used in the selection test was used.

The data were subjected to regression analysis using the SISVAR statistical program, version 5.4 (Ferreira, 2011). The maximum lethal concentration was then estimated from the derived regression equation, determining the selected value in the evaluated range.

Evaluation of density and pH

The density of the poultry litter substrate was evaluated to determine whether this interfered with the action of the NEPs. For this, we weighed 1 L of each batch of litter (new and old) and used the results is based on the quotient of both.

The method used to determine the pH of the litter was the same as for soils (Claessen, Barreto, Paula, & Duarte, 1997); three repetitions were performed for each lot. Each repetition included 10 cm^3 of the bed taken in an Erlenmeyer flask (10 cm^3 of bed) to which 25 mL of 0.01 M $CaCl_2$ solution was added, stirred for 15 min at 250 rpm, and kept to rest for 30 min before the pH was determined. The potentiometer was first calibrated with buffer solutions of pH 7.0 and pH 4.0 before determining the pH of the samples.

Evaluation of the effect of bed on the pathogenicity and virulence of NEPs

The non-native species, *S. feltiae* and *S. carpocapsae*, and the native isolate *H. amazonensis* UEL 08 were evaluated, with a view to low performance or UEL 07 without test testing. The JIs were applied to the new and old litter, and a 3 × 2 factorial design was used for this evaluation (three NEPs and two types of bedding). In all procedures, a concentration of 100 JIs/cm² was used, and the control was treated with water alone. For each treatment, 10 repetitions were made with 15 adult insects, totaling 150 insects per treatment. Each repetition corresponded to a plastic pot with a metallic mesh glued to the lid to ensure aeration. The volume of the pot was approximately 2 L, with a 12 cm diameter base, and 113,097 cm² area. Each pot was filled to a height of 1.25 cm with the litter, which corresponded to 50 g for the first batch litter (lighter) and 100 g for the fifth batch litter (heavier). These were then moistened with 45 mL of tap water.

The pots were kept in an air-conditioned chamber at 25 ± 1 °C and without photoperiod. The evaluation was performed after five days, when the numbers of dead and live insects were noted to estimate the percentage of mortality. The dead insects were then dissected to confirm the mortality from NEPs.

The data were subjected to normality and homoscedasticity tests, following which the medians were compared by the Tukey test using the SISVAR statistical program ($P \leq 0.05$) (Ferreira, 2011).

Results and Discussion

Selection of native isolates of entomopathogenic nematodes that affect Alphitobius diaperinus

Adults

It was found that all tested nematodes demonstrated pathogenicity in adults, with the exception of *S. brazilense* (IBCB-n 06), which did not differ from the control. A large variation in virulence was also observed, with mortality rates between 3.5% and 76.5%. The native species *Heterorhabditis* sp. (UEL 07) and *H. amazonensis* (RSC 05), as also the isolate *S. carpocapsae* (All), differed from the others and showed mortality rates above 66% (Table 2).

Table 2
Mortality (%) of *Alphitobius diaperinus* adults caused by entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis* spp.) under laboratory conditions (Temperature: 25 ± 1 °C, UR: 70 ± 10%, and without photoperiod)

Species (Treatments)	Isolates	Mortality (Average %)
Heterorhabditis amazonensis	UEL 07	76.50 ± 1.81 a
Heterorhabditis amazonensis	RSC 05	73.50 ± 5.47a
Steinernema carpocapsae	All	70.00 ± 1.55 a
Steinernema feltiae	IBCB-n 47	56.75 ± 3.87 b
Heterorhabditis sp.	IBCB-n 40	45.00 ± 5.51 b
Heterorhabditis sp.	ALHO-GL	45.00 ± 2.80 b
Heterorhabditis amazonensis	UEL 08	33.25 ± 2.37 c
Steinernema diaprepesi	AM 163	31.75 ± 5.27 c
Heterorhabditis indica	IBCB-n 05	31.50 ± 1.34 c
Heterorhabditis amazonensis	UEL 01	31.00 ± 3.81 c
Heterorhabditis sp.	IBCB-n 44	30.00 ± 3.81 c
Heterorhabditis sp.	JPM4	25.00 ± 5.51 c
Heterorhabditis sp.	IBCB-n 46	23.25 ± 5.57 c
Steinernema rarum	PAM	21.75 ± 9.71 c
Heterorhabditis sp.	NEPET11	21.75 ± 6.61 c
Steinernema brazilense	IBCB-n 06	3.50 ± 1.81 d
Control	-	0.00 ± 0.00 d
C.V.		22.44

* Averages followed by the same lowercase letter in the column did not differ significantly by the Scott-Knott test ($P \leq 0.05$).

It is possible to justify the low mortality observed for *S. brazilense* (3.50%) *and S. diaprepesi* (31.75%) as these nematodes have body lengths greater than 1 mm, which may hinder its penetration into the host body via natural openings (Román & Figueroa, 1994; Nguyen, Ginarte, Leite, Santos, & Harakava, 2010).

We also noted that there was a significant variation in the virulence of the nematodes that were tested. This can be attributed to several factors. Li, Liu, Lewis and Tarasco (2016) reported that larvae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), belonging to the same beetle family, when infected by NEPs, starts to produce detoxifying enzymes that can delay or inhibit or reduce the effect of these NEPs. However, this can vary depending on the nematode used and the species of insect it infects. Grewal, Nardo and Aguillera (2001) has highlighted the behavioral resources of both hosts and NEPs that affect the process of parasitism and the infection of the host, and these include, mainly, the ability to move and search for the host, ability to penetrate and provoke the infection, and ability to overcome defenses of the immune system (Lewis et al., 2006).

The authors Geden et al. (1985) and Szalanski, Palmer, Mckay and Steelman (2004) have pointed out that there may be variation in the virulence observed for the same species (*S. feltiae* and *S. carpocapsae*) on lesser mealworm. In this study, we observed a mortality rate of 70% in the adults infected by *S. carpocapsae* (all), which was similar to the value reported by Szalanski et al. (2004) for *S. carpocapsae* (UK, Kapow, Agriotos, Mexicano, Mexicano/Breton), but was well above that observed by L. F. A. Alves et al. (2005) and V. S. Alves et al. (2012) for the same species

In the present study, variations within the same species was also observed for *H. amazonensis* UEL 01, UEL 08, and UEL 07, for which we recorded adult mortality rates of 31%, 33.25%, and 76.5%, respectively, highlighting the importance of conducting resource selection tests.

Larvae

In general, when comparing larval mortality (Table 3) with adults (Table 2), there is a greater susceptibility of larvae to infections, with 12 out of 16 isolates showing more than 60% of mortality confirmed by NEPs. This greater susceptibility was also observed by Geden et al. (1985), Szalanski et al. (2004), and L. F. A. Alves et al. (2005). Several factors can justify this result—larvae show higher activity than adults, facilitating their encounter with the NEP; the larvae also show less rigidity when compared to adults, which facilitates the penetration of the JIs; in addition, during ecdysis, the higher exposure of the host beetle makes them more susceptible to infection by larvae.

Native individuals *Heterorhabditis* sp. (NEPET 11), *Heterorhabditis* sp. (UEL 07), *Heterorhabditis* sp. (IBCB-n 40)*, and Heterorhabditis* sp. (UEL 08) were more virulent in larvae compared to the others showing 100%, 93.7%, 85.5%*,* and 85.5% mortality, respectively (Table 3). Among the non-native species, *S. feltiae* (IBCB-n 47), showed the highest mortality (96.0%) in larvae.

Although *H. amazonensis* UEL 08 and NEPET 11 presented inferior virulence in the adult stage (21.75% and 33.25%, respectively) (Table 2), in the larvae, they were found to show high virulence. Therefore, it is suggested that these are not discarded for future tests, as they have been found effective in the control of the larval phase, and can interrupt the insertion cycle and consequently control population growth of the pest. Further, *S. diaprepesi,* and *Heterorhabditis* sp. (GL) also demonstrated opposite effects on larvae and adults (Table 3).

The mortality results observed in this study for *S. rarum* (MAP) were lower (27.25%) than those observed by Del Valle et al. (2016), who obtained values of up to 72.7%. Szalanski et al. (2004), studied the effect of different types of *S. feltiae* and *S. carpocapsae* on rat worm larvae, and reported mortalities ranging from 0% to 100% and from 16.8% to 93.2%, respectively.

Table 3
Mortality (%) of *Alphitobius diaperinus* larvae caused by entomopathogenic nematodes (*Steinernema* spp. and *Heterorhabditis* spp.) under laboratory conditions (Temperature: 25 ± 1 °C, UR: 70 ± 10%, and without photoperiod)

Species (Treatments)	Isolates	Mortality (%)
Heterorhabditis sp.	NEPET11	100.00 ± 0.00 a
Heterorhabditis amazonensis	UEL 07	93.75 ± 3.62 a
Steinernema feltiae	IBCB-n 47	96.00 ± 2.07 a
Heterorhabditis sp.	IBCB-n 40	85.50 ± 14.06 a
Heterorhabditis amazonensis	UEL 08	85.50 ± 3.66 a
Heterorhabditis sp.	IBCB-n 44	79.00 ± 2.07 b
Steinernema carpocapsae	All	77.00 ± 14.03 b
Heterorhabditis amazonensis	RSC 05	72.75 ± 10.27 b
Heterorhabditis amazonensis	UEL 01	70.75 ± 4.79 b
Heterorhabditis indica	IBCB-n 05	68.75 ± 9.63 b
Heterorhabditis sp.	JPM4	60.25 ± 4.04 b
Heterorhabditis sp.	IBCB-n 46	39.75 ± 2.01 c
Steinernema rarum	PAM	27.25 ± 7.80 d
Heterorhabditis sp.	ALHO-GL	19.00 ± 7.95 d
Steinernema diaprepesi	AM 163	16.75 ± 4.30 d
Steinernema brazilense	IBCB-n 06	0.00 ± 0.00 e
Control	-	0.00 ± 0,00 e
C.V.		22.44

* Averages followed by the same lowercase letter in the column did not differ significantly by the Scott-Knott test (P ≤ 0.05).

Concentration test using adult host

Based on the results from the selection tests, the non-native species *S. feltiae*, *S. carpocapsae* and the native *H. amazonensis* (UEL 07), were found to be most effective on adults, and for farmers preferring not to use chemical controls, these species show good multiplication capacity in the host (Rahoo, Mukhtar, Gowen, Rahoo, & Abro, 2017), which can guarantee their persistence in the environment.

The three individuals caused mortality in adults of *A. diaperinus* in all evaluated concentrations. From these results, we identified the best concentrations for use for each of the isolates.

Adult mortality from the application of *S. carpocapsae* (All) varied between 58.0% and 90.0% (Figure 1), where an application in the highest concentration (50.0 Jls/cm²) caused a higher mortality. In tests with *S. feltiae* (IBCB-n 47), mortality varied between 56.0% and 98.0% (Figure 2); the highest mortality was observed for concentrations of 30 and 40 Jls/cm². The

application of *H. amazonensis* (UEL 07) showed a variation in adult mortality between 20.0% and 46.0%, where the concentration of 40 Jls/cm² resulted in the maximum control.

Further, it was noted that the cascade mortality showed little variation between the concentration of 20 Jls/cm² for the isolate *S. carpocapsae* and 30 Jls/cm² for the isolate *S. feltiae*. According to Gaugler, Wang and Campbell (1994), these isolates can reach the minimum number of NEPs needed to infect and cause the death of the host.

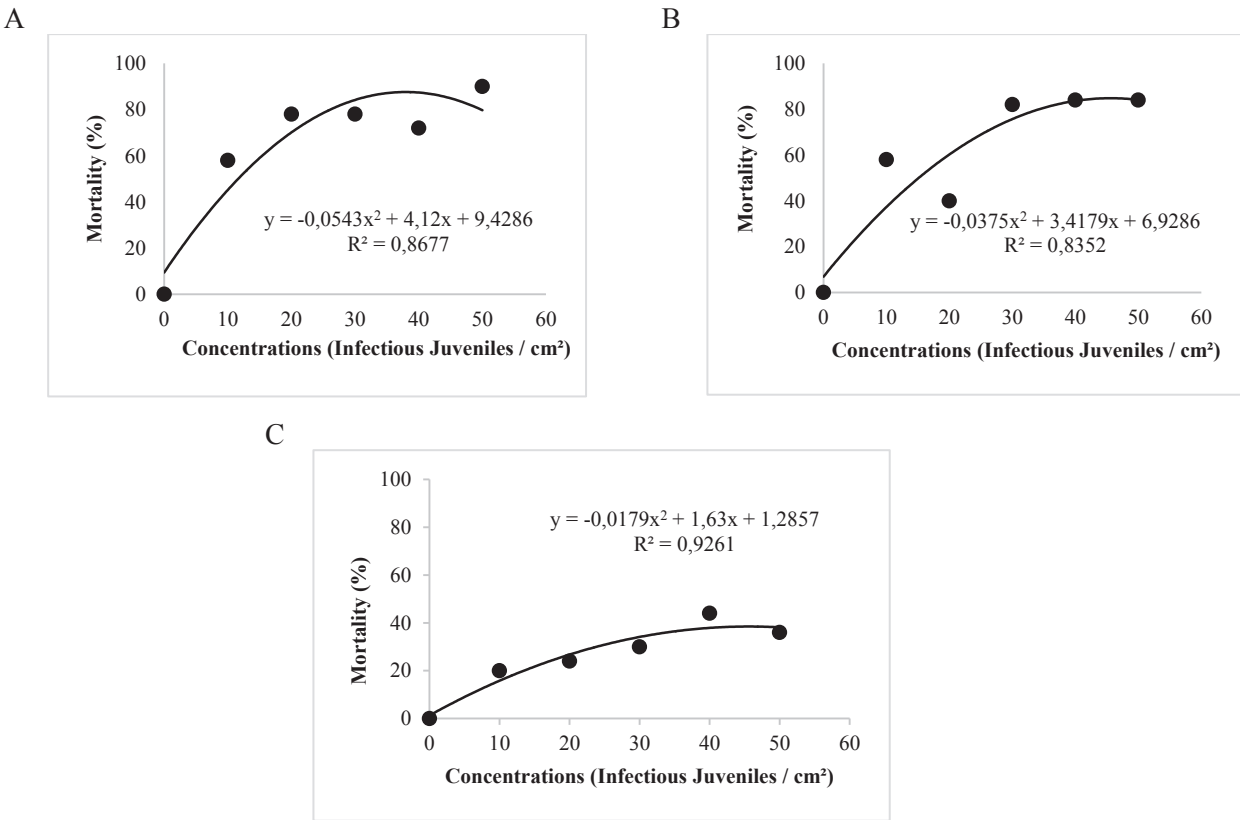

Figure 1. Mortality regression curve (%) of adults of *Alphitobius diaperinus* as a function of entomopathogenic nematode (NEP) concentrations (Jls/cm²): A) *Steinernema carpocapsae* (All), B) *Steinernema feltiae* (IBCB-n 47), and C) *Heterorhabditis amazonensis* (UEL 07). Maximum estimated mortality: A) 87.6% with application of 37.95 Jls/cm²; B) 84.81% with application of 45.57 Jls/cm²; and C) 38.48% with application of 45.64 Jls/cm².

We observed that the effect of *H. amazonensis* (UEL 07) we observed that the effect of *H. amazonensis* (UEL 07) was reduced when applied at low concentrations, mainly at 10 and 20 Jls/cm² (Figure 1), mainly at 10 and 20 Jls/cm² concentrations (Figure 1). When comparing these results with the selection test where concentrations of 100 Jls/cm² were used, the latter showed better efficiency (76.5% of average mortality). Rahoo et al. (2017) indicated that the probability of being infected may be related to the number of nematodes

applied, since the application of higher rates most often results in higher mortality.

Concentration test using larvae as host

We evaluated the effect of various concentrations on the mortality of larvae, and the results were similar to that observed for the adults. As in the previous test, all isolates caused mortality even at the lowest concentration. The results for larvae compared to adults (Figure 1) reinforce the idea that *A. diaperinus* larvae may be more susceptible to infection by NEPs (Figure 2).

Larval mortality as a function of the concentration caused by *S. carpocapsae* (All) was between 79.25% and 96.0%, with the highest percentage for an application of 30 JIs/cm². The isolate of *S. feltiae* (IBCB-n 47), as well as the *S. carpocapsae* (All), showed a mortality percentage above 80% in all evaluated samples, varying between 83.25% and 98.0%, with the highest percentage corresponding to the concentration of 50 JIs/cm². When analyzing the mortality regression curve for larvae for the two isolates (Figure 2), it can be observed that at the lowest concentration (10 JIs/cm²), both isolates showed a mortality rate greater than 79%, and the response due to increased concentration was not very variable.

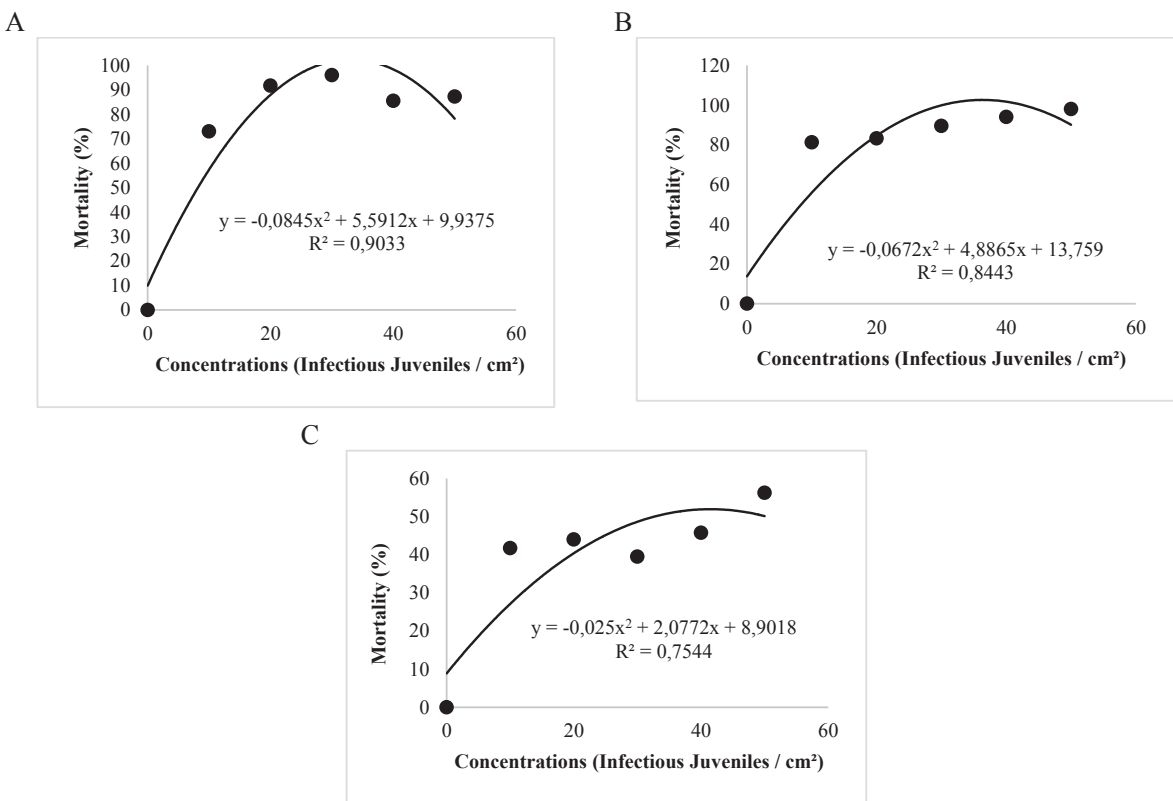

Figure 2. Mortality regression curve (%) of *Alphitobius diaperinus* larvae as a function of concentrations (JIs/cm²) of entomopathogenic nematodes: A) *Steinernema carpocapsae* (All) (JIs/cm²), B) *Steinernema feltiae* (IBCB-n 47), and C) *Heterorhabditis amazonensis* (UEL 07). Maximum estimated mortality: A) 102.42% with application of 33.08 JIs/cm², B) 102.83% with application of 36.46 JIs/cm², and C) 51.97% with application of 41.46 JIs/cm².

The UEL 07 isolate, similar to the results from the concentration test with adults, showed reduced performance when applied in the smallest concentrations. Thus, it is recommended that the largest number of JIs per area is used to ensure control of the pest.

These conclusions are based on tests done under controlled conditions. In tests undertaken under field-conditions, NEPs are likely to be subject to a series of deleterious factors, such as temperature and exposure to ammonia, in addition to having to search for the host (V. S. Alves, Moino, Santa-Cecilia, Andaló, & Souza, 2009). Hence, these conclusions should be taken as preliminary, requiring further corroboration from field-based studies."

Assessment of density and pH

The density of the new bed was 0.346 and that of the old bed was 0.726. This justified the use of half the quantity of litter in treatment with old beds compared to treatment with new beds to meet a depth of 1.25 cm in each experimental unit.

The pH of the new and old beds was 7.6 and 7.5, respectively. Thus, there was no change in the pH of the substrate between litter batches.

Evaluation of the effect of bed on pathogenicity and virulence of NEPs

There was no significant difference in the effect of each isolate when applied to new and old bedding. On the other hand, within each bed condition, there was a difference between the isolates (Table 4).

Table 4
Effect of new (first batch) and old (fifth batch) poultry litter on the virulence of entomopathogenic nematodes on the adults of *Alphitobius diaperinus* under laboratory conditions. Temperature: 25 ± 1 °C, without photophase (dark)

Treatments	Bed type	
	New	Old
Steinernema feltiae (IBCB-n 47)	60.7 ± 7.2 Aa*	80.0 ± 5.8 Aa
Steinernema carpocapsae (All)	58.,7 ± 9.9 Aa	74.7 ± 10.1 Aa
Heterorhabditis amazonensis (UEL 08)	20.0 ± 6.,7 Ba	28.7 ± 5.9 Ba
Control	0.0 ± 0.0 Ba	0.0 ± 0.0 Ca
C.V.	36.65	

* Averages followed by the same uppercase letter in the column and lowercase letter in the line do not differ from each other by Tukey's test (P ≤ 0.05).

In the treatment with new bed, *H. amazonensis* (UEL 08) did not differ from the control, reaching 20% mortality. *S. feltiae* and *S. carpocapsae* were the most virulent, with mortality rates of 60.7% and 58.7%, respectively, which did not differ from each other (Table 4).

In the treatment with old bed, all isolates differed from the control. *H. amazonensis* (UEL 08) was found to be the least virulent showing a mortality rate of 28.7%, while those of the non-native *S. feltiae* (IBCB-n 47) and *S. carpocapsae* (all) were found to be the most virulent, with average mortality values of 80% and 74.7%, respectively, which did not significantly differ from each other (Table 4).

As previously mentioned, V. S. Alves et al. (2012) evaluated the isolate *S. carpocapsae* (IBCB-n 02) under laboratory conditions of 25 ± 1 ºC and 12 hours of photophase, with the same concentration of JIs/cm² and found lower mortality (19%) compared to results from this study (74.7%).

The low virulence of the isolate *S. carpocapsae* (All) in the study by V. S. Alves et al. (2012) can be explained by the ambusher behavior of the nematode (Kaya & Gaugler, 1993). In addition, in that study, the nematodes were inoculated in sterile soil at the bottom of the experimental units, the poultry bed was then deposited on the soil, and the insects were introduced on the bed. Given the distance between the soil and the bed surface where the insects were present, the nematode had less contact with the insects, which could justify the lower mortality observed. Unlike the bioassays performed in this study, none of the nematodes were inoculated directly into the bed and in direct contact with the insects.

Few studies have evaluated the effect of different batches of aviary litter on the pathogenicity and virulence of entomopathogenic nematodes. Jeffrey (2001) reported that the pH of the chamber varied from 6.0 to 9.0. In this study, the pH varied between the two batches of poultry litter, from 7.6 (new litter) to 7.5 (old litter). In the study by Kung, Gaugler and Kaya (1990), the results indicated that the pH between 4.0 and 6.0 was ideal for the effectiveness of *S. carpocapsae* and *S. glaseri* and observed that the best rates were at pH 5.1, with 83% decrease in the host numbers. The performance was found to decrease slightly from pH 6.5. In our study, given the minimal change in the pH between new and old beds, the virulence of the nematodes was not affected.

The continuous process of poultry breeding, molting, and regrowth of feathers and recommencement of egg laying throughout the breeding cycle makes the bed microbiota very diverse. Physical variations in the bed, such as humidity, water activity, and pH, were observed by Paganini (2004), who concluded that the higher the humidity, the greater the water activity and the higher the pH.

Despite presenting virulence above 80% in laboratory conditions, the native isolates (UEL 07 and UEL 08) reduced performance both in the concentration test and in the interference test of the poultry litter, when compared to the two non-native isolates, suggesting that the exotic isolates have the greatest potential for use in the field. However, the use of exotic species should be avoided, as these can become a threat to native species and could also affect non-target species (Dolinski & Moino, 2006).

The native individuals UEL 07 and UEL 08 belong to the species *H. amazonensis* (Guide, 2019), which currently include at least six isolated species in Brazil (Andaló, Nguyen & Moino, 2006; Barbosa-Negrisoli et al., 2010; Brida et al., 2017; Guide, 2019). Thus, it is suggested that the other isolates that were not considered in this study are also tested for their virulence and other parameters such as recommended lethal concentrations and the effect of bed conditions are determined. Further, evaluations in the field for all isolates need to be carried out. In addition, studies on the manipulation of native species are suggested, wherein improvements based on a combination of resources that may differ from already tested combinations should be explored for better performance.

Conclusion

The *H. amazonensis* UEL 07 and UEL 08 were the native isolates with the greatest potential for controlling *A. diaperinus*.

The exotic nematodes *S. feltiae* (IBCB-n 47) and *S. carpocapsae* (All) were more virulent at lower concentrations, and also when applied to different types of bedding, when compared to native *H. amazonensis* UEL 07 and UEL 08.

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