






**BIOLOGICAL ACTIVITY OF ENTOMOPATHOGENIC
NEMATODES ON *Anastrepha fraterculus*
(DIPTERA: TEPHRITIDAE)**

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Abstract

Anastrepha fraterculus (Wiedemann) is one of the main pests of fruit farming, and entomopathogenic nematodes (EPNs) represent an important control tool of this species. Thus, the objective of this study was to evaluate the biological activity of different isolate against *A. fraterculus* larvae and adults. Bioassays were performed using a suspension of three isolates of *Heterorhabditis amazonensis* IBCB 24, *Steinernema carpocapsae* IBCB 02 and *Steinernema feltiae* IBCB 47 at six concentrations (control - without nematodes), 50, 150, 300, 500, 1000 and 1500 infective juveniles (IJs)/mL of water per 3^o instar larvae. It was verified the susceptibility of larvae of *A. fraterculus* to isolates of EPNs and a significant increase of the pupal mortality in the function of the concentration of IJs inoculated by larva (above 75%). After the dissection of pupae and adults of *A. fraterculus* from infected larvae, the concentration of 1500 IJs/mL of EPNs provided the highest rate of multiplication of IJs by insect, equating to maximum concentration tested 1500 IJs/mL. Adults of *A. fraterculus* from larvae infected with EPNs longevity of five days, being less than adults from uninfected larvae by IJs (135 days). *H. amazonensis* IBCB 24, *S. carpocapsae* IBCB 02, and *S. feltiae* IBCB 47 proved to be promising as agents of biological control of *A. fraterculus*.

Keywords: Biological control. Infective Juvenile. Heterorhabditidae. Steinernematidae.

1. Introduction

The South American fruit fly *Anastrepha fraterculus* (Wiedemann 1830) (Diptera: Tephritidae) is among the main fruit-growing pests in South America, spread from the southern United States to northern Argentina. In the national territory, it occurs in 23 states, and establishes relations with more than 100 species of plants, causing losses of around US\$ 120 million/year (Zucchi 2017). The damage generated occurs directly, during egg-laying and larval development in the fruit, and indirectly, because they facilitate the entry of secondary pathogens through holes from egg-laying (Dias et al. 2013). In Brazil, current management strategies against *A. fraterculus* include a spray application of phosphorus and pyrethroids insecticides (Harter et al. 2015). However, worldwide studies have shown that using toxic baits can be a management option for infested areas (Navarro-Llopis et al. 2012; Borges et al. 2015).

Considering the great threat that *A. fraterculus* imposes to fruit crops, and the intense contamination provided by the current control technique, it is fundamental to study alternative methods of control, within the context of Integrated Pest Management (Garcia et al. 2017). In this sense, the use of entomopathogenic

nematodes (EPNs) is considered a highly promising method (Dias et al. 2008; Soliman et al. 2014; James et al. 2018), due to the active search of the host in the soil, and the mutual association with *Xenorhabdus* (Thomas and Poinar) and *Photorhabdus* (Boemare, Louis and Kuhl) bacteria (Dowds and Peters 2002; Poinar and Grewal 2012; Labaude and Griffin 2018). Infective juveniles (IJs) of EPNs enter the host through natural openings or through the cuticle and release the bacteria into the hemocoel, where they reproduce and kill the host by septicemia within 24 to 48 hours, making the environment favorable for the development and reproduction of nematodes (Lewis et al. 2006; Poinar and Grewal 2012; Subramanian and Muthulakshmi 2016). The genus *Anastrepha* has been shown to be susceptible to EPNs, as observed in *Anastrepha obliqua* (Macquart) (Diptera: Tephritidae), *Anastrepha serpentina* (Wiedemann) (Diptera: Tephritidae) and *Anastrepha ludens* (Loew) (Diptera: Tephritidae) exposed to *Heterorhabditis bacteriophora* (Poinar) (Lezama-Gutiérrez et al. 2006; Toledo et al. 2006; Toledo et al. 2014), *Anastrepha suspensa* (Loew) (Diptera: Tephritidae) to *S. feltiae* (Heve et al. 2016) and *A. fraterculus* to *H. bacteriophora* RS88, *S. riobrave* RS59, *Heterorhabditis amazonensis* IBCBn 24 and *Oscheius* sp. (Barbosa-Negrisoni et al. 2009; Foelkel et al. 2017). However, for the successful use of these agents in the management of fruit flies, it is essential to assess the pathogenicity of EPNs in the laboratory to select the best lethal concentrations that can be used in the field (James et al. 2018; Labaude and Griffin 2018; Chergui et al. 2019). Therefore, in this study, we aimed to evaluate the biological activity of entomopathogenic nematodes against *A. fraterculus* larvae and adults.

2. Material and Methods

Breeding and maintenance of *A. fraterculus*

Specimens of *A. fraterculus* were obtained from the Insect Biology Laboratory of the Eliseu Maciel College of Agronomy (UFPEL) and the entomopathogenic nematode isolates from the collection of entomopathogenic organisms of the “Oldemar Cardim Abreu Collection” of the Biological Institute of São Paulo, State of São Paulo, Brazil. The culture of *A. fraterculus* was maintained in an air-conditioned environment (25±1°C), with relative humidity (RH) 70 ± 10% and photophase of 12h. The insects were reared on artificial diet, following the methodology of Salles (1992) and adapted by Nunes et al. (2013).

Maintenance and multiplication of species of EPNs

The nematodes *Heterorhabditis amazonensis* (Andaló) IBCB 24, *Steinernema carpocapsae* IBCB 02 and *Steinernema feltiae* IBCB 47 were multiplied in larvae of *Galleria mellonella* (Linnaeus) (Lepidoptera: Pyralidae) from fourth to the fifth instar, according to the methodology of Machado (1988). For the multiplication of the nematodes, five *G. mellonella* larvae were placed in Petri dishes (9 cm in diameter) lined with two sheets of filter paper, moistened with 1.5 mL nematode suspension at 500 IJs/cm², (100 IJs/larvae). Petri dishes were capped and sealed with polyvinyl chloride (PVC) film and stored at 25±2°C and RH > 80%. After three days of mortality, dead larvae were transferred to White's trap (White 1927) and kept at 25±1°C and RH at 70±10% until the emergence of IJs. Collected IJs were kept in distilled water (1 cm deep) in Erlenmeyer flasks maintained at 18±1°C, 70±10% RH, and used 48h after collection.

Biological Activity Against *A. fraterculus* Larvae

To verify the activity of entomopathogenic nematodes (EPNs) on third instar larvae of *A. fraterculus*, three EPNs isolates (*H. amazonensis* IBCB 24, *S. carpocapsae* IBCB 02 and *S. feltiae* IBCB 47) were used. Therefore, 3rd instar larvae of *A. fraterculus* were placed in plastic containers (200 mL) containing 100 grams of sterilized fine sand, 10% moisture. After penetration of *A. fraterculus* larvae in the sand (20 to 30 s), isolate suspensions (treatments) of *H. amazonensis* IBCB 24, *S. carpocapsae* IBCB 02 and *S. feltiae* IBCB 47 were inoculated with a graduated glass pipette (5 mL). For each EPNs isolate, seven concentrations were used: zero (control - without nematodes), 50, 150, 300, 500, 1000, and 1500 IJs/mL of water per larvae. After inoculation, the containers were sealed with perforated lids (approx. 1 mm diameter) for aeration and maintained in a climatized room at 25 ± 1°C, 70 ± 10% relative humidity and photophase of 12h. Evaluations were performed daily until the emergence of adults. Pupae mortality was evaluated at three days after inoculation of the entomopathogenic nematodes. Based on the results, were to estimate the concentration

required to kill 50% and 90% of exposed pupae [Lethal concentration (LC), LC_{50} and LC_{90} , respectively]. As well as pupae and adults of *A. fraterculus* were dissected using an entomological clamp to quantify the number of IJs to determine the nematode multiplication rate per insect with the aid of a stereoscopic microscope (40×). The bioassay was conducted in a completely randomized design, with three treatments and five replicates per concentration, with 10 third instar larvae of *A. fraterculus* per concentration ($n=50$).

Biological Activity Against *A. fraterculus* Adults Subsection

For this purpose, was used the same methodology as the previous bioassay. To evaluate the biological activity against *A. fraterculus* adults, LC_{50} e LC_{90} values were used estimated in the concentration-response curves. The adults that emerged after the treatment were transferred to cages, consisting of 500 mL plastic pots with the bottom lined with voile (5cm), and were fed on a solid diet based on soybean extract, wheat germ and sugar (3: 1: 1) (Nunes et al. 2013). Water was provided in polyurethane sponges inserted in 9-cm diameter Petri dishes. The bioassays were maintained in a climatized room at $25 \pm 1^\circ\text{C}$, $70 \pm 10\%$ relative humidity, and photophase of 12h. Inviabile pupae were removed from the treatments on the third day after the emergence of the last adult. The biological parameter evaluated was the longevity (days) of adults of *A. fraterculus*. The bioassay was conducted in a completely randomized design, with three treatments (EPNs isolate) and five replicates with 10 third instar larvae of *A. fraterculus* per concentration LC_{50} or LC_{90} ($n=50$).

Statistical analysis

Generalized linear models of the exponential family of distributions (Nelder and Wedderburn 1972) were used for the analyses of studied variables (pupae mortality (%), number of juveniles for pupae and adults and longevity of adults). When there were significant differences among treatments, multiple comparisons (Tukey's test, $P < 0.05$) were performed using the `glht` function by means of a `Multcomp` package, with adjustment of p-values. All analyses were performed using the "R" statistical software version 2.15.1 (R Development Core Team 2012). A binomial model with a complementary log-log link function (gompit model) was used to estimate the lethal concentrations (LC_{50} and LC_{90}), using the Probit Procedure in the software SAS version 9.2 (SAS Institute, 2008).

3. Results

We observed a significant increase ($P < 0.05$) in the susceptibility of *A. fraterculus* larvae depending on the concentration of EPNs isolate evaluated, *S. carpocapsae* IBCB 02 ($F= 27.15$; d.f= 6; $P > 0.001$), *H. amazonensis* IBCB 24 ($F= 33.455$; d.f= 6; $P > 0.001$) and *S. feltiae* IBCB 47 ($F= 38.715$; d.f= 6; $P > 0.001$), providing high pupae mortality (approximately 80%) at the maximum concentration tested (1500 IJs/ml) (Figure 1). However, the isolated *S. carpocapsae* IBCB 02, the concentration of 300 IJs/ml was significantly similar ($P = 0.05$) the maximum concentration evaluated, 1500 IJs/ml (Figure 1). As well, it was verified for the isolated *S. feltiae* IBCB 47 at the concentration of 1000 IJs/ml, equating to be statistically ($P = 0.05$) to the concentration of 1500 IJs/ml (Figure 1).

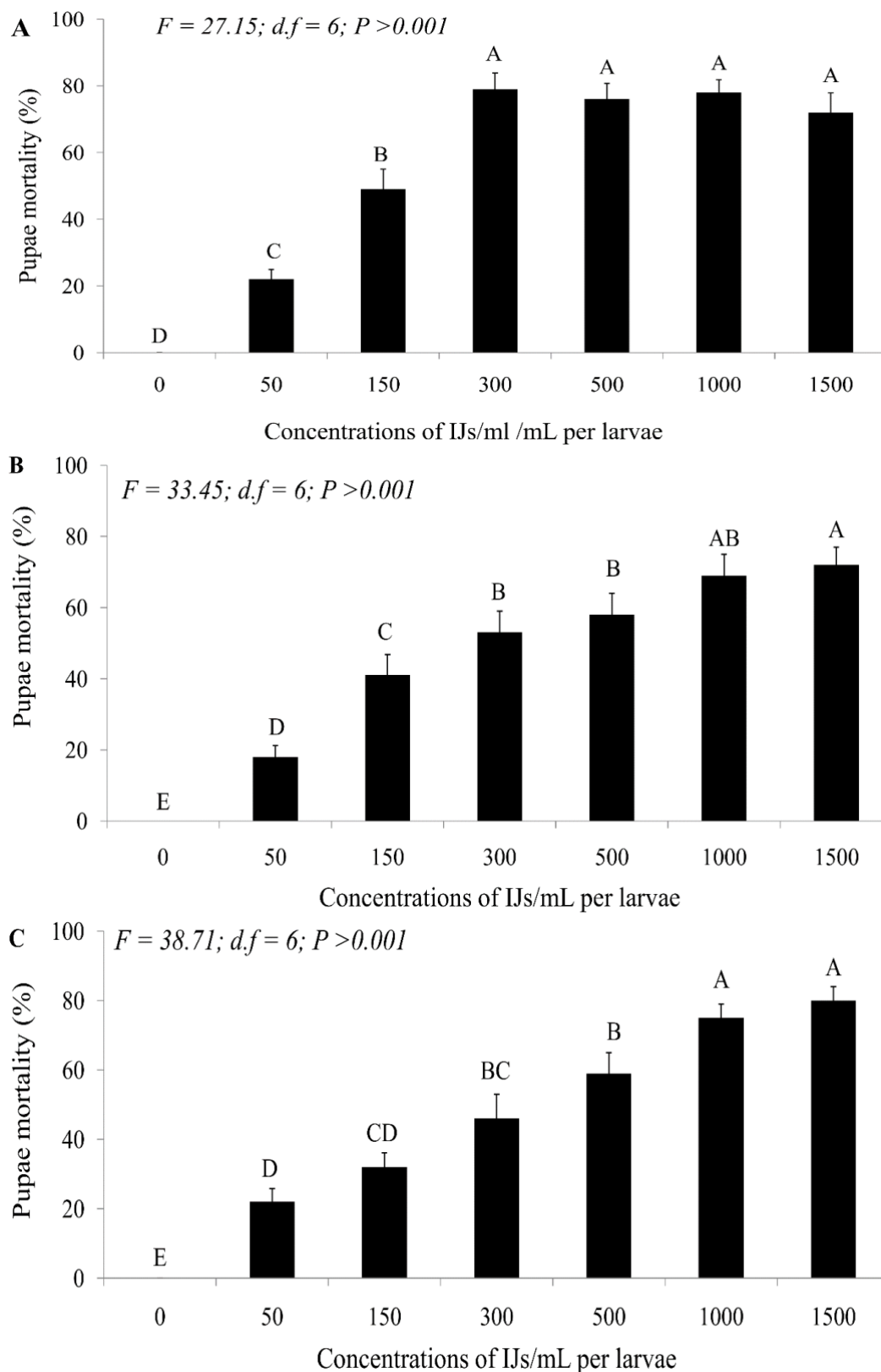


Figure 1. Pupae mortality (percentage \pm standard error) of *A. fraterculus* after application to concentrations of EPNs isolates in the third instar larvae. A – *Steinernema carpocapsae* IBCBn 02; B – *Heterorhabditis amazonensis* IBCBn 24; C – *Steinernema feltiae* IBCBn 47. *Bars (\pm SE) with the same letter (for each EPNs isolate) are not significantly different (GLM with quasi-binomial distribution followed by Tukey’s post hoc test; $P < 0.05$).

It was observed a high rate of multiplication of EPNs in the hemocoel of insects through the process of dissection of pupae ($F = 38,715; d.f = 6; P > 0,001$) and adults ($F = 14,609; d.f = 6; P > 0,001$) of *A. fraterculus* (Table 1). It was found that the concentration of 500 IJs/ml, provided for all isolates, rates of multiplication of the EPNs significantly similar when compared to the maximum concentration tested (1500 IJs/ml) (Table 1).

Table 1. Multiplication rate (number average \pm Standard error) of Infective Juvenile in pupae and adults of *Anastrepha fraterculus* in different concentrations.

Concentration of IJs	Pupae	Adults
<i>Steinernema carpocapsae</i> IBCBn 02		
Control	0.00 \pm 0.00 ^C	0.00 \pm 0.00 ^D
50	1.93 \pm 0.85 ^B	1.21 \pm 0.11 ^C
150	2.87 \pm 0.86 ^{AB}	1.22 \pm 0.11 ^C
300	2.00 \pm 0.80 ^B	1.83 \pm 0.31 ^B
500	2.71 \pm 0.99 ^{AB}	2.71 \pm 0.99 ^A
1000	2.40 \pm 0.80 ^{AB}	2.40 \pm 0.84 ^A
1500	3.64 \pm 0.29 ^A	2.55 \pm 2.79 ^A
<i>Heterorhabditis amazonensis</i> IBCBn 24		
Control	0.00 \pm 0.00 ^C	0.00 \pm 0.00 ^D
50	2.20 \pm 0.48 ^B	0.39 \pm 0.13 ^D
150	2.44 \pm 0.5 ^B	1.28 \pm 0.47 ^C
300	2.77 \pm 0.90 ^B	2.73 \pm 0.44 ^B
500	5.34 \pm 1.36 ^A	3.29 \pm 0.79 ^{AB}
1000	4.83 \pm 0.34 ^A	3.21 \pm 3.90 ^{AB}
1500	5.98 \pm 0.24 ^A	4.52 \pm 2.12 ^A
<i>Steinernema feltiae</i> IBCBn 47		
Control	0.00 \pm 0.00 ^D	0.00 \pm 0.00 ^D
50	1.09 \pm 0.66 ^C	0.65 \pm 0.15 ^C
150	2.13 \pm 0.75 ^{BC}	2.64 \pm 0.93 ^B
300	3.35 \pm 0.53 ^B	2.50 \pm 0.78 ^B
500	4.01 \pm 0.73 ^{AB}	4.45 \pm 0.89 ^A
1000	4.65 \pm 1.07 ^{AB}	3.06 \pm 0.22 ^{AB}
1500	5.90 \pm 0.75 ^A	3.87 \pm 1.32 ^A

Averages number with the same letter (for each EPNs isolate) are not significantly different (GLM with quasi-binomial distribution followed by Tukey's post hoc test; $P < 0.05$).

Based on the concentration-response curves from the bioassays, the data were adjusted to the proposed model (chi-square values < 10) (Table 1). In relation to the median lethal concentration (LC₅₀ and LC₉₀) and based on the overlap of the confidence intervals (CI), the EPNs *S. carpocapsae* IBCB 02, *H. amazonensis* IBCB 24 and *S. feltiae* IBCB 47 did not differ among themselves, with values of CL₅₀ ranging from 427.2 (326.2 - 524.8 IJs/ml) to *S. carpocapsae* IBCB 02 to 636.9 (515.0 - 738.5 IJs/ml) for *H. amazonensis* IBCBn 24 (Table 2). Similarly, it has been observed for the values of CL₉₀, which varied from 1551.4 1768.8 1388.9 (- IJs/ml) to *S. feltiae* IBCB 47 to 1828.0 2145.4 1604.4 (- IJs/ml) for *H. amazonensis* IBCB 24 (Table 2).

Table 2. Estimate of the LC₅₀ and LC₉₀ (IJs/mL) as well as the confidence interval (CI 95%) of entomopathogenic nematodes to third instar larvae of *Anastrepha fraterculus*.

Isolate	Slope \pm SE	LC ₅₀ (CI 95%) ^a	LC ₉₀ (CI 95%) ^b	χ^2 ^c	d.f. ^d
<i>Steinernema carpocapsae</i> IBCBn 02	3.56 \pm 0.40	427.2 (326.2 - 524.8)	1703.0 (1480.4 - 2028.8)	3.57	6
<i>Heterorhabditis amazonensis</i> IBCBn 24	2.19 \pm 0.19	636.9 (515.0 - 738.5)	1828.0 (1604.4 - 2145.4)	7.85	6
<i>Steinernema feltiae</i> IBCBn 47	2.82 \pm 0.22	595.0 (519.2 - 676.8)	1551.4 (1388.9 - 1768.8)	2.81	6

^{a,b}LC₅₀ and LC₉₀ – lethal Concentration of entomopathogenic nematodes (IJS/mL) required to kill 50% or 90% of *A. fraterculus* pupae, respectively (CI: confidence interval at 95% error probability); ^c χ^2 – Pearson's chi-square value; ^dd.f. – degrees of freedom.

In addition, adults of *A. fraterculus*, from third instar larvae infected with *S. carpocapsae* IBCBn 02, *H. amazonensis* IBCBn 24 and *S. feltiae* IBCBn 47 at concentrations CL₅₀ and CL₉₀, estimated from the concentration-response curves showed average longevity of approximately 5 days, for both the CL₅₀ (F = 31.64; df = 3; P < 0,001) and CL₉₀ (F = 22.12; df = 3; P < 0,001), differing statistically from adults from larvae without the presence of IJs (control treatment) that showed average longevity of 135 days (Figure 2).

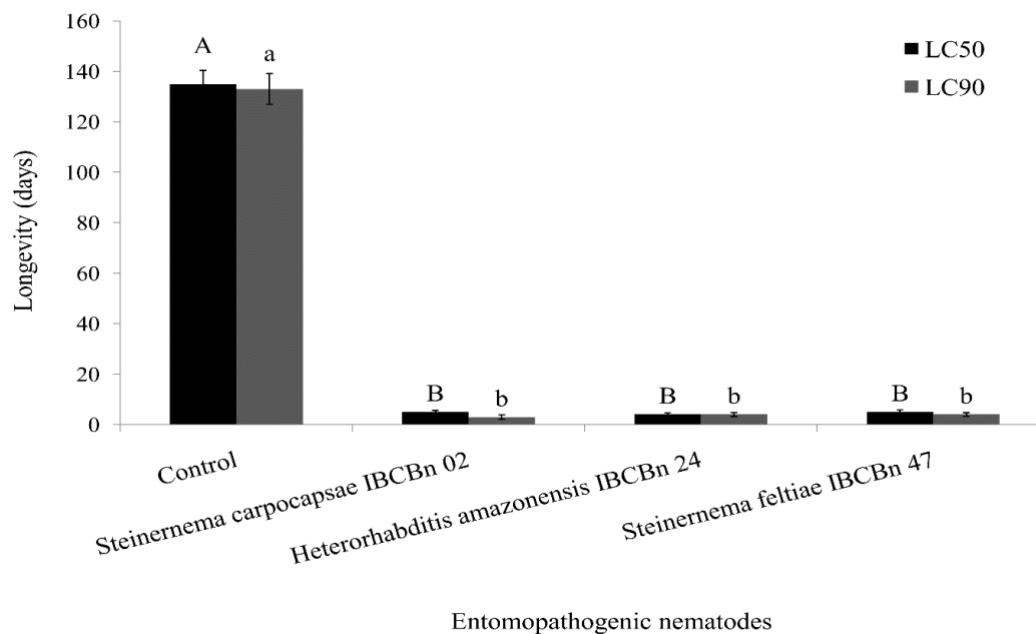


Figure 2. The longevity of adults of *A. fraterculus* after application of EPNs isolate in LC₅₀ e LC₉₀ values estimated from the concentration-response curves. “Bars (± SE)” followed by the same color and different letters differ significantly by the Tukey test ($P > 0.05$) for values of CL₅₀ and CL₉₀.

4. Discussion

The use of entomopathogenic nematodes has already been used in the management of different species of fruit flies (Dias et al. 2008; James et al. 2018). In the present study the susceptibility of *A. fraterculus* to EPNs was verified, corresponding to results of previous studies, which observed the efficiency of different isolates in the control of immature insect phases of the genus *Anastrepha* (Barbosa-Negrisoni et al. 2009; Foelkel et al. 2017). When compared of genus *Heterorhabditis*, the differences in the pathogenicity of the genus *Steinernema* can be explained by variations in the strategies of the host or from the entrance of the IJs in the insect due to penetrating the interior of the insects occur through the mouth, anus, blowholes and track cuticle (Lewis et al. 2006).

Among the pupae mortality rates reported in this study, the highest average occurred for *S. feltiae* IBCB 47 (80.0%) at the concentration of 1500 IJs/ml. This result is due to the searching efficiency of the IJs of this isolate, which uses mechanisms of recognition of the host by chemical compounds, moving to this, or increasing in the substrate as a way to carry out the infection (Grewal et al. 2001). In contrast, the isolate that provided the lowest average pupal mortality was *H. amazonensis* IBCB 24, with 72.0% at the highest concentration (1500 IJs/ml). The low efficiency of higher concentration may be related to specific characteristics of the isolated, because for some the inoculation of high dosages can generate intraspecific competition in search of the host, thereby reducing the infectivity (Dias et al. 2008).

The rates of multiplication of the isolates in the hemocoel of insects (pupae and adults) were affected by the concentrations of inoculated, the larger ones were obtained in the intermediate concentration (500 IJs/larvae). These results indicate a reduction in the multiplication of IJs in high concentrations, which occurs because of the limited number of resources available in the insect host (Dias et al. 2008). The efficiency of the multiplication of isolated demonstrates the ability of the IJs to remain in the environment and carry out the control for a longer period, which emphasizes the importance of the adequacy of the concentrations of IJs used (Jackson 1999). In addition, the behavior of the isolates may have contributed to reducing virulence, as each species behaves differently, allowing a high or low infection in the host, which is linked to the size and structure of the insect, which can lead to failure the time of infection and consequently reducing the mortality rate (Brida et al. 2017).

The lethal concentration (LC₅₀ and LC₉₀) was obtained in smaller quantities of IJs to the isolated *S. carpocapsae* IBCB 02 and *S. feltiae* IBCB 47, with 427.2 IJs/ml and 1551,4 IJs/ml, respectively. The efficiency of control these isolated from insects of the genus *Anastrepha* was also reported by Foelkel et al. (2016)

indicating 90.0% mortality in larvae of *A. fraterculus* (314.7 IJs/larva) and for Heve et al. (2016), with 85.0% of mortality by *S. carpocapsae* (200 IJs/larva) in *Anastrepha suspensa* (Loew) (Diptera: Tephritidae).

Another factor evaluated in the present study was the emergence of adult insects infected with EPNs and consequent reduction in their longevity due to infection. When penetrating the insect's integument, IJs usually cause mortality between 24 and 48 hours, however, the emergence of infected adults indicates an insect resistance to infection during the pupal period (Rodrigues-Trentini 1996). This resistance is due to its immune system, which synthesizes genes encoding antimicrobial peptides that encapsulate and inactivate the pathogen (Castillo et al. 2011; Strand 2008). The synthesis of these genes in fruit flies was observed in adults of *Ceratitis capitata* (Wiedemann) after infection by *S. carpocapsae*, which produced specific genes, aiming at the inhibition of the microorganism (Tavares 2011). Inactivation of the pathogen during the pupal phase of the insect and reestablishment of its activity during the adult phase indicate the ability of these organisms to overcome the insect's immune system. These they secrete antibiotic molecules that inhibit the action of the encapsulation enzyme produced by the host, conferring resistance to the process of phagocytosis (Vallet-Geley et al. 2008).

For the first time, it observed the effect of IJs of EPNs in newly emerged adults after treatments with different isolates in the immature phases. The EPNs drastically reduced the longevity of *A. fraterculus* adults, causing mortality before completing the pre-oviposition period, which would prevent them from causing fruit damage (Zart et al. 2010). Although the inoculated concentration is of great importance for the success of the infection rate (Dias et al. 2008), it was verified that, regardless of the concentration used (CL₅₀ or CL₉₀ values), IJs were able to resist the insect's immune system mortality in a short period.

The present study deals with the high pathogenicity of the EPNs isolates *S. carpocapsae* IBCB 02, *H. amazonensis* IBCB 24, and *S. feltiae* IBCBn 47 to *A. fraterculus*, which demonstrated efficiency in the different concentrations of IJs used. Another factor assessed was the efficiency of multiplication of the isolates in the hemocoel of insects, which underscores the ability of the IJs to survive in the environment, multiply and carry out the control of a greater number of hosts. This research also reports the ability of IJs to remain in adults of *A. fraterculus* after emergence, which is reported for the first time in the insect. The insect's ability to emerge even if infected may be related to the action of its immune system, which can slow down the infectious process. However, even emerging, the insects would be prevented from causing damage, since they died between the first and fourth day of life, long before they reached the pre-oviposition period.

In Brazil, the search for EPNs -based products with the potential for pest control is growing annually (Brida et al. 2017). Thus, the availability of biological products based on nematodes can assist in the integrated management of *A. fraterculus*, mainly, after studies characterize the natural occurrence of these two genera in fruit orchards in Brazil (Barbosa-Negrissoli et al. 2009). However, it is important to continue the studies in the field, once which, the environmental parameters such as temperature, moisture, vegetation types, and soil properties can affect the survival and virulence of nematodes in the field. However, based on the results, we can conclude that the *H. amazonensis* IBCB 24, *S. carpocapsae* IBCB 02, and *S. feltiae* IBCB 47 isolates may be a future alternative for an integrated management program of *A. fraterculus*.

5. Conclusions

The isolates *Steinernema carpocapsae* IBCBn 02, *Heterorhabditis amazonensis* IBCBn 24 and *Steinernema feltiae* IBCBn 47 are pathogenic to larvae and pupae of *A. fraterculus*, in addition to causing fly mortality, they reduce the longevity of adults, preventing them from causing damage before they reach them. the pre-oviposition period, contributing to the management of this pest and with promising potential as agents for the biological control of *A. fraterculus*.

Authors' Contributions: CHANEIKO, S.M.: acquisition of data, analysis and interpretation of data, and drafting the article; BRIDA, A.L.: acquisition of data, analysis and interpretation of data, drafting the article, and critical review of important intellectual content; BERNARDI, D.: analysis and interpretation of data, drafting the article, and critical review of important intellectual content; LEITE, L.G.: drafting the article; GARCIA, F.R.M.G.: analysis and interpretation of data, drafting the article, and critical review of important intellectual content. All authors have read and approved the final version of the manuscript.

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Ethics Approval: Not applicable.

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