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Article | DOI: 10.21307/jofnem-2018-039

JOURNAL OF NEMATOLOGY
Issue 3 | Vol. 50 (2018)

Responses of *Anastrepha suspensa*, *Diachasmimorpha longicaudata*, and Sensitivity of Guava Production to *Heterorhabditis bacteriophora* in Fruit Fly Integrated Pest Management

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This paper was edited by Raquel Campos-Herrera.

Received for publication April 14, 2018.

Abstract

Caribbean fruit fly, also known as Caribfly or *Anastrepha suspensa*, is a major tephritid pest of guavas. A virulent entomopathogenic nematode (EPN) species was investigated to suppress the fruit-to-soil stages of Caribflies, which are also attacked by the koinobiont parasitoid *Diachasmimorpha longicaudata* in south Florida. The main objective was to develop a feasible and cost-effective EPN-application method for integrated pest management (IPM) of Caribfly to improve guava production. Naturally infested guavas were treated with increasing *Heterorhabditis bacteriophora* infective juvenile (IJ) concentration or rate (0, 25, 50, …, 1,600 IJs cm^{-2}) in field trials to measure the optimum IJ rate and then examine sensitivity of producing guavas to inclusion of *Heterorhabditis bacteriophora* in Caribfly IPM plans. Relative survival of Caribfly in treatments significantly decreased with increasing IJ rate from 0 to 100 IJs cm^{-2}. Similarly, probability of observing large numbers of parasitoid wasps (*Diachasmimorpha longicaudata*) in EPN treatments significantly declined with increasing IJ rate (0–100 IJs cm^{-2}), even though the non-target effects of *Heterorhabditis bacteriophora* on relative survival of *Diachasmimorpha longicaudata* could not be determined because of few emerging parasitoid wasps. Optimum suppression (≥ 60%) of Caribfly was consistently achieved at 100 IJs cm^{-2} or 17,500 IJs $fruit^{-1}$. Profitability analysis showed that *Heterorhabditis bacteriophora* can be included in Caribfly IPM tactics to produce guavas. Costs of EPNs in Caribfly IPM are minimized if *Heterorhabditis bacteriophora* is strategically applied by spot treatment of fruit. Repayment of costs of EPN-augmentation by spot treatments appears achievable by recovering 5.71% of the annual yield losses (≥1,963 kg ha^{-1} ≈ US$ 8,650 ha^{-1}), which are largely due to Caribfly infestation. Hectare-wide EPN-augmentation (or broadcasting) method requires more fruit recovery than the total annual yield losses to repay its high costs. Profitability of guava production in south Florida will not be very sensitive to marginal costs of the spot treatment method, when compared to the field-wide broadcasting of *Heterorhabditis bacteriophora*.

Key words

Biological control, *Heterorhabditis bacteriophora*, *Anastrepha suspensa*, *Diachasmimorpha longicaudata*, Cost minimization strategy, Amortization, Feasible Caribfly IPM, *Psidium guajava*.

Caribbean fruit fly (Caribfly; *Anastrepha suspensa* Loew) causes quality damage and blemishes to guava (*Psidium guajava* L.) when adult female Caribfly deposits eggs into ripening guavas. After larvae hatch, the developing third instar larvae migrate from infested fruit that has dropped to the ground and quickly pupate in soil. Following eclosion of pupae, adult Caribfly emerges from soil (Heve et al., 2017a).

The aerial spray of insecticides against adult Caribfly in infested orchards will not control Caribfly in infested fruit or soil. Bagging of guavas on trees, or destruction of infested guavas on the ground to kill *Anastrepha suspensa*, is not often observed across guava orchards in south Florida because of much labor and expenses involved. Moreover, any developing useful parasitoids, for example *Diachasmimorpha longicaudata* Ashmead (Braconidae), of tephritid pests in infested guavas will be killed if Caribfly-infested guavas on the ground are destroyed with the intention to manage Caribfly in orchards. Mass-trapping is not practiced in south Florida, perhaps, because of lack of strong powerful attractants to sufficiently bait large numbers of adult Caribfly. For unknown reasons, attempt to use the sterile insect release method against Caribfly is not observed in south Florida. Currently, no techniques are used to manage "fruit-to-soil" stages of Caribfly, similar to the way entomopathogenic (EPN) species have successfully been used to control soil-borne insect pests in glasshouse and the field (Grewal et al., 2005; Lacey and Georgis, 2012; Miles et al., 2012). At least 75% suppression of insect pests have been reported following application of EPNs to manage the corn earworm (*Helicoverpa zea* Boddie), the corn rootworms (*Diabrotica* spp.), the *Diaprepes* root weevils on citrus, and the sweet potato weevil (*Cylas formicarius* Fabricius), among numerous insect pests whose larvae migrate and pupate in soils (Grewal et al., 2005; Dolinski et al., 2012; Lacey and Georgis, 2012; Miles et al., 2012). Several reports have also shown that infective juveniles (or IJs) of numerous species of the genera *Heterorhabditis* and *Steinernema*, tested against fruit flies in laboratory and field conditions, successfully killed 50% to 90% of the olive fruit fly (*Bactrocera oleae* Rossi), the Medbfly (*Ceratitis capitata* Wiedemann), the Caribfly, and other tephritid pests (Sirjani et al., 2009; da Silva et al., 2010; Minas et al., 2016; Heve et al., 2017a, b, 2018; Torrini et al., 2017).

The biology of EPN species makes them useful for biological control of soil-borne insect pests (Grewal et al., 2005; Labaude and Griffin, 2018). After locating insect hosts, EPNs penetrate the host through the spiracles, anus, and mouth, and then release symbiotic antibiotic-producing bacteria from their gut into the infected insects (Grewal et al., 2005; Poinar and Grewal, 2012; Stock et al., 2017; Labaude and Griffin, 2018). The toxins produced by the bacteria kill the infected insects (Stock et al., 2017; Labaude and Griffin, 2018). While inside a cadaver or the EPN-killed insect, the nematodes feed on the internal parts of the cadaver and reproduce in large numbers. The free-living stage (infective juvenile or IJ progenies) of the EPNs emerge as food resources diminish, and then quickly spread in soils in search of new hosts to infect. Because of these specific behaviors, EPNs killed the fruit-to-soil stages of Caribfly in laboratory and field trials (Heve et al., 2017a, b, 2018).

According to the observations by Laznik et al. (2012) or Laznik and Trdan (2014), the aerial spray of insecticides will not significantly affect the activities of EPN species against Caribfly in soils. Combination of any management approaches, that will kill adult Caribfly aboveground, and application of EPN which kills Caribfly at its "fruit-to-soil stages" should control Caribfly pest better. However, such an integrated fruit fly control approach merits study because the costs of including EPN species in the Caribfly integrated pest management (IPM) plans will increase total costs of producing guava fruit. Also, the rapid development of susceptible third instar larvae to long resistant pupal stage confers the ability of Caribfly to escape EPN infections, requiring high EPN-application rates to adequately suppress Caribfly (Heve et al., 2017a, 2018). Therefore, we hypothesized that application of EPNs at high IJ rates in Caribfly IPM requires a more cost-effective EPN-augmentation strategy to make it economically feasible for guava production in south Florida.

Under laboratory conditions, the efficacy of virulent EPNs to Caribfly increased with increasing rates of virulent EPN IJs and then declined after efficacies reached maximum (Heve et al., 2017a). This suggests that studies are needed to estimate an EPN-application rate that will maximize suppression of Caribfly in the field. In recent field trials, *Heterorhabditis bacteriophora* (Poinar) effectively suppressed Caribfly better than seven other multiple EPN species treatments achieved (Heve et al., 2017b, 2018). Therefore, *Heterorhabditis bacteriophora* appeared to be more promising to manage Caribfly in south Florida (Heve et al., 2018). On the other hand, female parasitoid wasps (*Diachasmimorpha longicaudata*) oviposit eggs into Caribfly larvae in fruit for development of their progenies to adult or wasps. Larvae of tephritid pests in fruit parasitized by *Diachasmimorpha longicaudata* are killed at pupal stage in soil (Vargas et al., 2012; Weems et al., 2014; Schliserman et al., 2016; Simmonds et al., 2016). In the field, releases of large

numbers of female wasps (*Diachasmimorpha longicaudata*) significantly suppressed *Anastrepha* fruit flies in orchards (Sivinski et al., 1996; Montoya et al., 2000). But recent field trials showed that progenies of both Caribfly and *Diachasmimorpha longicaudata* developing in naturally infested guavas decaying on the ground will certainly receive EPN treatments if EPN species are applied to suppress Caribfly in orchards (Heve et al., 2017b, 2018).

In this study, we examined the response of *Anastrepha suspensa* and that of its parasitoid *Diachasmimorpha longicaudata* in naturally infested fruit over a range of *Heterorhabditis bacteriophora* IJ rates. We measured the response of *Diachasmimorpha longicaudata* to *Heterorhabditis bacteriophora* in the field, because the wasps are endemic in south Florida orchards and are associated with incidence of Caribfly in infested guava fruit (Heve et al., 2017b, 2018). Our main objectives were to: (i) generate information from *Heterorhabditis bacteriophora* IJ dose-response study in the field to estimate annual costs of EPN-augmentation, (ii) examine the sensitivity of the guava production to the inclusion of *Heterorhabditis bacteriophora* in Caribfly IPM, and (iii) identify and suggest a feasible strategy to apply *Heterorhabditis bacteriophora* against *Anastrepha suspensa* in south Florida guava orchards.

Materials and methods

EPN-dose response studies in the field

Location and experimental design for field trials

A non-host avocado orchard at the coordinate (25°30′36.45″N, 80°30′10.74″W), belonging to the Tropical Education and Research Center of UF/IFAS in Homestead in south Florida, was used for field trials. The field in the avocado orchard was used to measure efficacy of EPN applied over infested guavas since variability between treatments could be more observed if the experiments were conducted on the ground in infested guava orchards, where soils were patchily infested by Caribfly (Heve et al., 2017b, 2018). Moreover, *Rockdale* soils across orchards in south Florida have similar physico-chemical properties such as high Ca content, high pH, and shallow hard rocky plow-pan with high drainage, among others (Li, 2015; Heve et al., 2018). A total of 40 cone-shaped screen-cages (base diameter = 80-100 cm; height = 50 cm) were placed singly beneath the canopy of an avocado tree in a randomized complete block design layout within 5 rows of avocado trees (Heve et al., 2018). The average distance between successive cages within a row of avocado trees was 17 m, whereas the distances between rows of the trees were *ca.* 23 m.

Inoculum

Infective juveniles (IJs) of *Heterorhabditis bacteriophora* were freshly produced in dark condition (Shapiro-Ilan et al., 2015), using the EPN-killed larvae of *Galleria mellonella* (L.) in the White trap method (Shapiro-Ilan and Gaugler, 2002). Without exposure to light, the IJs were stored in 3.78-litre-containers containing ample tap-water at 10 °C for 7 days before being used. The stock IJ suspensions were concentrated to determine and re-adjust the initial IJ density to 5,600 IJs ml^{-1} before IJs were immediately applied (Heve et al., 2017a, b, 2018).

Infested guavas and EPN treatments in the field

The periods from May to July were considered appropriate to measure efficacy of *Heterorhabditis bacteriophora* in the field, because largest numbers of adult Caribflies occurred in June-July, when highest soil temperatures were observed in south Florida (Heve et al., 2018, https://fawn.ifas.ufl.edu). At least 80 infested guava fruit (from different varieties of guava trees) on the ground were transferred from Caribfly-infested orchards to the cages in the avocado orchard on June 16, 2017. To increase the probability of observing sufficient numbers of emerging adult Caribflies with emerging parasitoid wasps (*Diachasmimorpha longicaudata*) in EPN treatments, two guava fruit were placed in a plot (circular area = 350 cm^2 soil surface) under each labeled cage: the numbers of emerging adult Caribfly or parasitoids do not differ significantly between sets of equal numbers of infested guava fruit (Heve et al., 2018). Four days later, each of the IJ concentrations or rates 0, 25, 50, 100, 200, 400, 800, and 1,600 IJs cm^{-2} of *Heterorhabditis bacteriophora* was measured in 100 ml of tap-water and then spread to evenly cover the two infested guava fruit in the plot (i.e., 350 cm^2 soil surface), using a 100-ml-measuring cylinder (Heve et al., 2018). An additional 20 ml of tap-water was used to rinse the cylinder and then added to the treatment, thereby, adjusting total volume of tap-water to be 120 ml in each replicate of each IJ rate (Heve et al., 2017b, 2018). Control (or 0 IJs cm^{-2}) was 120 ml of tap-water only (Heve et al., 2018). Five replicates of *Heterorhabditis bacteriophora* treatments at each IJ rate were made in plots. On the

7th day after the date of EPN application, pieces of yellow sticky traps were inserted in cages: adult Caribfly and parasitoid wasps, that emerged from soils in the 3rd to 4th weeks after fruit were added to plots, were trapped and monitored daily (Heve et al., 2018). On July 15, 2017, another 80 Caribfly-infested fruit were transferred from the guava orchards to cages in the avocado orchard, and 2 infested fruit cage^{-1} were added to each plot and then treated with freshly produced active *Heterorhabditis bacteriophora* IJs, using procedures described above.

Data analysis

Observations

The patterns of numbers of emerging Caribfly and those of the surviving parasitoid *Diachasmimorpha longicaudata* in the EPN treatments were examined using bar graphs. Data of both emerging adult Caribfly and parasitoid wasps (*Diachasmimorpha longicaudata*) from experiments repeated once in plots maintained under cages from June to August 2017 were pooled similar to the analysis in report of Hajihassani et al. (2013).

Relative survival of Caribfly

Pooled numbers of emerging Caribfly in replicates were converted into values from 0 to 1, according to equation 1 (Gotelli and Ellison, 2013):

$$Q = \frac{X}{Y} \quad (1)$$

where X is the density of emerging adult Caribfly observed in each replicate of each EPN treatment and Y is the replicate of a treatment with the highest number of adult flies in the pooled data. The relative survival of Caribfly was calculated or normalized in distribution by dividing values of Q in replicates of treatments by the arithmetic mean value ($\hat{u}$) of Q in replicates of the control.

Probability analysis for emerging parasitoid wasps

Based on assumption of Poisson 'probability' distribution for random variable outcomes, with reference to a mean outcome in each treatment (Gotelli and Ellison, 2013), the probability of observing a few parasitoid wasps in replicates of each *Heterorhabditis bacteriophora* IJ rate was calculated, using the equation 2:

$$q = \left(\frac{\lambda^N}{N!}\right)\left(e^{-\lambda}\right) \quad (2)$$

where e is a constant for the base of natural logarithm = 2.71828, λ is the mean number of surviving parasitoid in each treatment (or at each IJ application rate) and N is the numbers of the parasitoid wasps (*Diachasmimorpha longicaudata*) observed in a replicate of each EPN treatment. Therefore, the probability of observing a large number of emerging parasitoid wasps in treatments of *Heterorhabditis bacteriophora* was calculated as in equation 3:

$$p = 1 - q \quad (3)$$

Post-hoc test, regression and correlation

The R-software (R.3.3.2; R core group in Vienna, Austria) was used for all statistical analyses. One-way analysis of variance (ANOVA) was fitted in Tukey's HSD test at $P \leq 0.05$ to compare differences between values (means ± standard errors) of accumulated densities of emerging Caribfly or parasitoid wasps observed at IJ application rates. Regression analysis was used to establish the functional predicting model relating relative survival of Caribfly or the probability (p) for large numbers of parasitoid wasps (*Diachasmimorpha longicaudata*) to range of increasing IJ rate that controlled Caribfly. Moreover, association between emergence of adult Caribfly and that of the parasitoid *Diachasmimorpha longicaudata* in treatments (0–1,600 IJs cm^{-2}) was examined using Pearson correlation coefficient.

Annual cost estimates: observations and assumptions

In this study, 500 guava trees (1.28 ha)$^{-1}$ observed in the representative guava orchards were assumed to be similar across other orchards. The hour-labor wage considered was US$ 11.00 (Garcia et al., 2014). Though fewer hours of labor costs ha^{-1} may be required, we assumed a maximum of 4-hour-labor costs ha^{-1} for EPN-augmentation over individual fruit on the ground on any given day in the period March-July. The periods from March to July of each year were considered, because 64% to 70% of yearly accumulated population densities of emerging adult Caribflies (five guavas)$^{-1}$ have been observed in March-July 2017 (Heve et al., 2017b, 2018). Moreover, few fruit are observed on the ground on any given day from March to July, because guava trees in south Florida do not produce large numbers of guavas in

March-July when compared to August-February each year (Garcia et al., 2014; Evans et al., 2018), thereby, making EPN-augmentation by spot treatments possible to minimize costs. On any given day, the fruit that fell on the ground in March-July 2017 were assessed to be in ratios between 0 and 5 guavas tree^{-1} (Heve et al., 2017b). Though large numbers of fruit are not to be observed on the ground from March-July each year in south Florida, we estimated the average costs of *Heterorhabditis bacteriophora* IJs– ha^{-1} by extrapolating the numbers of guavas on the ground to 10 fruit tree^{-1} (Table 1). A representative commercial price of *Heterorhabditis bacteriophora* product available in the U.S.A is US$ 97 per 500 million nematodes. The frequency of monthly EPN-augmentation considered for the periods from March to July is five times year^{-1}. Each guava grower was assumed to apply active *Heterorhabditis bacteriophora* IJs at the best IJ rate that achieved maximum control of Caribfly in the trials reported here. The cost estimates are summarized in the footnote of Table 1.

Sensitivity analysis for *Heterorhabditis bacteriophora* in Caribfly IPM to produce guava

All costs, yearly wholesale base yield, average total annual yield, wholesale base price, overhead expenses, costs of disease, and pest management approaches, uncertainties, interest rate on capital (5%) and varieties of guavas among other factors in the reports of Garcia et al. (2014) and Evans et al. (2018) were considered and maintained on the assumption that a guava grower will likely combine both existing Caribfly control strategies and the use of *Heterorhabditis bacteriophora* against Caribfly. The annual costs (in Table 1 with footnote) required to apply *Heterorhabditis bacteriophora* were included in the existing Caribfly pest management costs which increased total costs of producing guavas in south Florida (Table 2). The annual net income after the inclusion of costs of using *Heterorhabditis bacteriophora* in Caribfly IPM was then compared to the current annual base net income in reports of Evans et al. (2018) (Table 2). The corresponding annual marketable guava yields required to repay costs of applying *Heterorhabditis bacteriophora* were estimated and the possibility of recovering these yields (for repayment of costs) from the annual guava yield losses were examined, based on assumption that EPN-augmentation will cause a retrieval of uninfested ripening guava yield from the losses to defray costs of EPN-augmentation.

Results

Effects of increasing IJ rate of *Heterorhabditis bacteriophora* on emergence of adult Caribfly in field trials

The numbers of emerging adult Caribfly observed in EPN treatments in each period are summarized in Figure 1A. During June-July 2017, densities of emerging Caribfly observed remained relatively constant (i.e., 17 adult flies emerged from two fruit plot^{-1}) over the IJ rates from 0 to 50 IJs cm^{-2} (Fig. 1A). But when the EPN treatments were repeated to newly collected two fruit plot^{-1} in the July-August 2017, numbers of the flies were reduced better than there were emerging adult flies in June-July 2017 (Fig. 1A). At 100 IJs cm^{-2}, *Heterorhabditis bacteriophora* consistently suppressed *ca*. 60% of emerging adult flies in plots from June to August 2017 (Fig. 1A). Consequently, relative survival of Caribfly from two fruit added to plots significantly decreased as application rate of *Heterorhabditis bacteriophora* IJs was increased in treatments (Fig. 2). The minimum numbers of emerging Caribfly were achieved at 100 IJs cm^{-2}, which is equivalent to EPN treatments to a single fruit in a plot (i.e., 350 cm^2) at '(50 IJs cm^{-2} spread over 350 cm^2 ≈ 17,500 IJs fruit^{-1})' (Fig. 1A). Additional increase in IJ rates higher than 100 IJs cm^{-2} tended to increase densities of emerging Caribfly (*Anastrepha suspensa*), even though treatments at 800 IJs cm^{-2} effectively suppressed Caribfly similar to treatments at 100 IJs cm^{-2} (Fig. 1A).

Emergence of the parasitoid *Diachasmimorpha longicaudata* following *Heterorhabditis bacteriophora*-augmentation over Caribfly-infested guava fruit in the field

The patterns of densities of emerging parasitoid wasps observed in the EPN treatments with increasing IJ rate are in Figure 1B. Parasitoid wasps were observed in all EPN treatments, except the treatments at the rates 800 IJs cm^{-2} (only in July-August 2017) and 1,600 IJs cm^{-2} (Fig. 1B). The emergence of the parasitoid wasps (*Diachasmimorpha longicaudata*) was directly related to the emergence of adult Caribflies in EPN treatments with increasing *Heterorhabditis bacteriophora* rate (Figures 1A,B, 3) because the linear relationship (Pearson correlation coefficient, r = 0.358; P = 0.023) observed between the accumulated densities of surviving parasitoid wasps and those of emerging adult Caribfly in plots was significant

Table 1. Cost estimates for the possible EPN-augmentation approaches in guava orchards to achieve maximum suppression of Caribfly at the best IJ rate[a].

Strategic EPN-augmentation option	In series, number of fruit tree^{-1} on the ground	Total number of fruit on the ground in 1 hectare[b]	Required IJs ha^{-1} of *Heterorhabditis bacteriophora*	Estimated cost of total number of IJs (US$ ha^{-1})
Hectare-wide EPN-augmentation or broadcasting at '(50 IJs cm^{-2} spread over a fruit in 350 cm^2)' seems to be less cost-effective	na	na	5×10^9	970[c]
Applying *Heterorhabditis bacteriophora* IJs over each infested fruit on soil surface at 17,500 IJs fruit^{-1} seems to be more economical	≤1	≤500	8,750,000	1.70
	2	1,000	17,500,000	3.40
	3	1,500	26,250,000	5.10
	4	2,000	35,000,000	6.79
	5[e]	2,500	43,750,000	8.49
	6	3,000	52,500,000	10.19
	7	3,500	61,250,000	11.88
	8	4,000	70,000,000	13.58
	9	4,500	78,750,000	15.28
	10	5,000	87,500,000	16.98
	The average costs of *Heterorhabditis bacteriophora* IJs ha^{-1} in each month			9.34[d]

na: Not applicable, because guava fruit that drop to the ground are not observed in every 1 cm^2 soil surface in infested guava orchards; [a]The equivalent best IJ rate for EPN treatment to an infested fruit in plot (i.e., 350 cm^2) at 50 IJs cm^{-2} is 17,500 IJs fruit^{-1} (Figures 1, 2); [b]Additional 28% was included to account for unexpected uncertainties or increases that may rarely occur from March to July; [c]Annual cost estimates for hectare-wide EPN-augmentation or broadcasting strategy in the period from March-July = 970 × 5 ≈ US$ 4,850 ha^{-1}; [d]Average annual cost estimates for spot treatment method in March-July period = (9.34 + four-hour-labor costs) × 5 ≈ US$ 266.70 ha^{-1}; [e]From March to July, the ratios between 0 and 5 fruit tree^{-1} on any given day will often be observed on the ground in guava orchards (Heve et al., 2018).

(Fig. 3). Though numbers of emerging wasps were not enough to sufficiently predict linear relationship between relative survival of the parasitoid wasps (*Diachasmimorpha longicaudata*) and IJ rate, the probability of observing large numbers of emerging parasitoid wasps significantly declined with increasing IJ rate from 0 to 100 IJs cm^{-2} (Fig. 4).

Effects of *Heterorhabditis bacteriophora*-augmentation on profitability of producing guavas in south Florida

The cost estimates for the possible EPN-augmentation strategies are summarized in footnote of Table 1 and then used for cost-benefit analysis in Table 2. For

Table 2. Cost-benefit analysis showing changing annual base net income level in response to varying total costs following inclusion of *Heterorhabditis bacteriophora* in Caribfly IPM plans to suppress Caribfly (*Anastrepha suspensa)* better in south Florida guava orchards.

		Methods to include *Heterorhabditis bacteriophora* in Caribfly IPM	
	Before inclusion of *Heterorhabditis bacteriophora* in IPM	Spot treatment technique	Hectare-wide broadcasting
Details	US$ ha^{-1}	US$ ha^{-1}	US$ ha^{-1}
1. Production costs[a]			
Irrigation	494	494	494
Fertilizers	1,359	1,359	1,359
Insecticides	1,112	1,112	1,112
Herbicides	1,112	1,112	1,112
Fungicides	988	988	988
Heterorhabditis bacteriophora (in Table 1)	0	267	4,850
Labor (pruning; supervision; others)	1,606	1,606	1,606
Interest on capital (5%)	334	347	576
2. Fixed costs			
Cash overhead:			
Insurance	247	247	247
Taxes	247	247	247
Non-cash overhead:			
Land rent	1,236	1,236	1,236
Other overhead	1,483	1,483	1,483
3. Harvesting and marketing costs			
Picking and sales cost	6,919	6,919	6,919
4. Total costs	17,137	17,417	22,229
5. Returns on 'wholesale base yield'			
Annual wholesale base revenue[b, c]	39,537	39,537	39,537
Annual base net income[d]	22,400	22,120	17,308

[a]*Heterorhabditis bacteriophora* and insecticides were considered to effectively suppress fruit-to-soil stages of Caribfly belowground and their adults aboveground, respectively, because other Caribfly control approaches are rarely observed in south Florida guava orchards due largely to high costs involved; [b]Annual wholesale base revenue = 'Annual wholesale base yield (ca. 8,967 kg ha^{-1}) times base price (US$ 4.41 kg^{-1})' is equivalent to US$ 16,000 acre^{-1} in report of Evans et al. (2018); [c]These are largely non-susceptible mature hard dark green guavas; susceptible "light-green-to-yellow" ripening guavas lost annually are estimated to be (10,930 kg ha^{-1} minus ca. 8,967 kg ha^{-1} ≈ 1,963 kg ha^{-1}), equivalent to US$ 8,650 ha^{-1}; [d]Additional information can be obtained from reports of Garcia et al. (2014) and Evans et al. (2018).

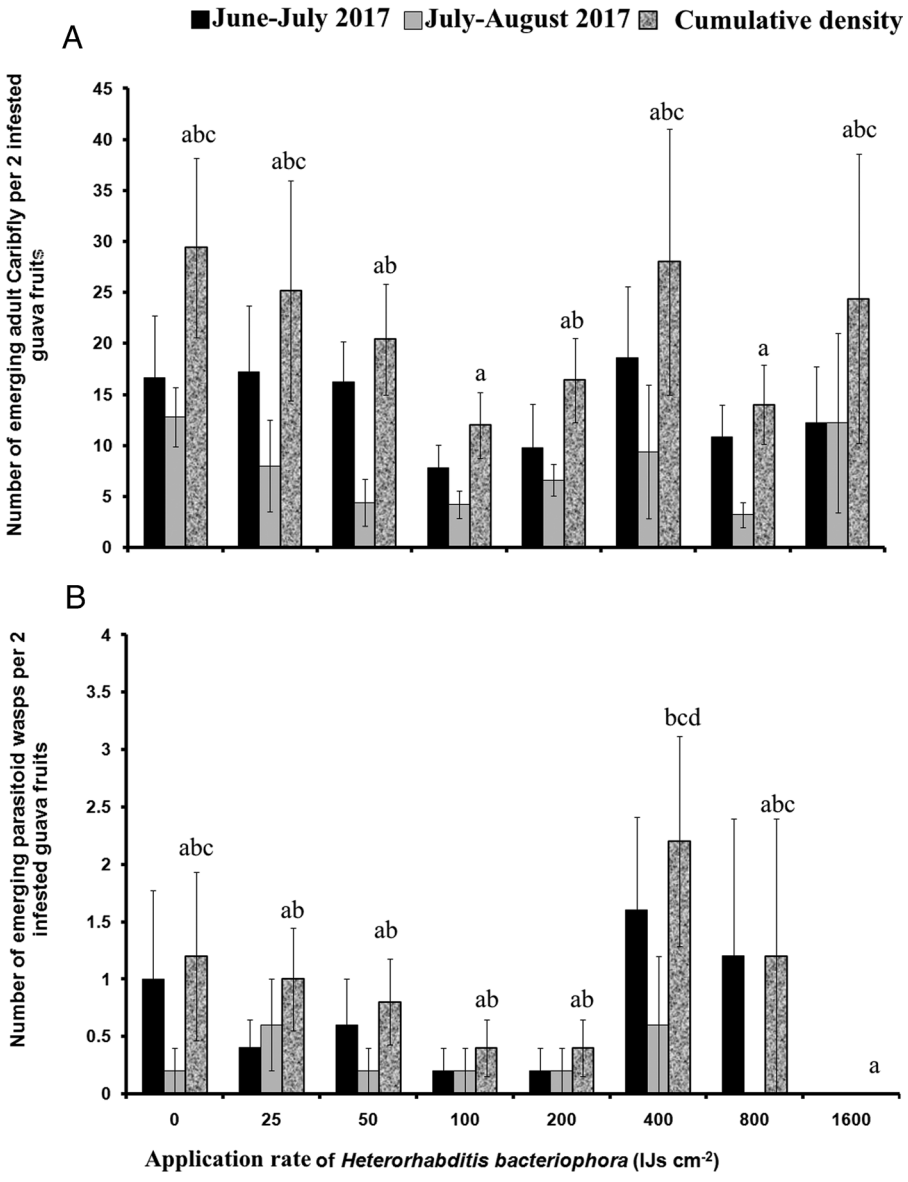

Figure 1: The patterns of numbers of emerging (A) adult Caribfly and (B) parasitoid wasps (*Diachasmimorpha longicaudata*) observed in EPN treatments, with increasing IJ rate of *Heterorhabditis bacteriophora* applied over two Caribfly-infested guavas in the field. Tukey's HSD tests at $P \leq 0.05$: same letter on top of bars (± standard errors) denotes no significant differences, even though numerical margins were observed, in accumulated densities of emerging adult Caribfly or the parasitoid wasps between application rates of *Heterorhabditis bacteriophora*.

the case where *Heterorhabditis bacteriophora* at the optimum IJ rate 17,500 IJs fruit^{-1} is applied over each Caribfly-infested fruit that drops from guava trees to the ground in the periods from March to July, the annual base net income (or base profit) will decrease by 1.25% (i.e., from US$ 22,400 ha^{-1} to US$ 22,120 ha^{-1}) (Table 2). But for the case where hectare-wide EPN-augmentation strategy is used to apply *Heterorhabditis bacteriophora* IJs at the equivalent rate '(50 IJs cm^{-2} applied in plot (i.e., 350 cm^2))' in the periods from March to July, the annual base net income (US$ 22,400 ha^{-1}) will reduce by *ca.* 22.75% to US$ 17,308 ha^{-1} (Table 2). The net profit margins will still be very high if either the field-wide EPN-augmentation or

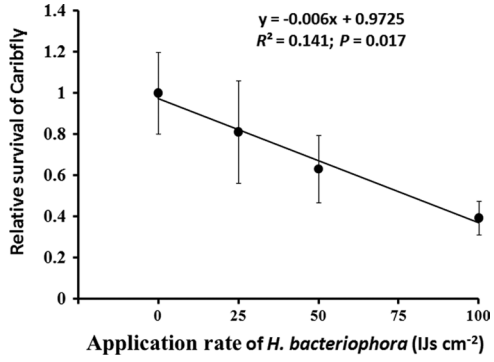

Figure 2: Relationship between relative survival of Caribfly and increasing IJ rate of *Heterorhabditis bacteriophora* used to treat two infested guavas plot^{-1} in field trials from June to August 2017. The coefficient of determination and the probability statistic for significance level are R^2 and P, respectively.

the spot treatment method is used to augment *Heterorhabditis bacteriophora* to target Caribfly in infested guava orchards, from March to July.

Nevertheless, the quantity of marketable guavas that will be required to amortize the costs of applying *Heterorhabditis bacteriophora* in spots is estimated to be 112 kg ha^{-1} (or 1.25% of the annual wholesale base yield (8,967 kg ha^{-1})), whereas 2,063 kg ha^{-1} of saleable guavas will be needed to pay off the costs of EPN applied by field-wide EPN-augmentation (i.e., broadcasting method). Based on the average total

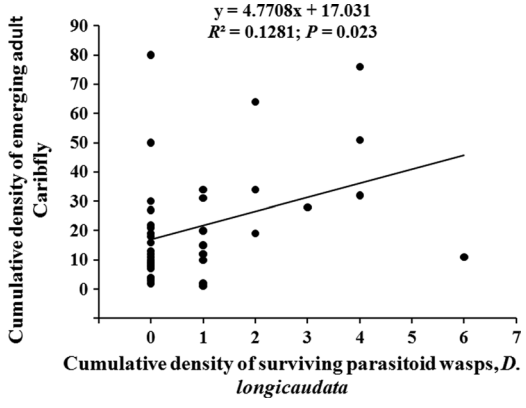

Figure 3: Linear relationship between cumulative densities of emerging adult Caribfly and those of the surviving parasitoid wasps, *Diachasmimorpha longicaudata*. The coefficient of determination is R^2 whereas P is the probability statistic for significance level.

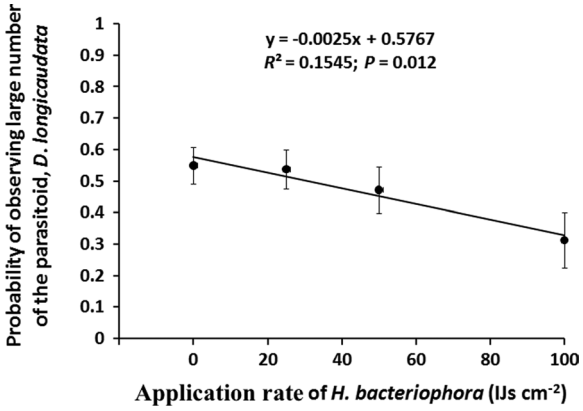

Figure 4: Relationship between probability of observing large numbers of the parasitoid *Diachasmimorpha longicaudata* and the increasing IJ rate from 0 to 100 IJs cm^{-2}, following EPN-augmentation of *Heterorhabditis bacteriophora* over two guavas in field trials from June to August 2017. The coefficient of determination is R^2, whereas the probability statistic for significance level is P.

annual guava yield of 10,930 kg ha^{-1}, the annual yield losses are estimated to be 1,963 kg ha^{-1} (in footnote of Table 2). Assuming the required amounts of saleable uninfested ripening fruit to repay costs are to be recovered from the yearly yield losses following EPN-augmentation, then only the full repayment of costs of augmenting *Heterorhabditis bacteriophora* by spot treatment will feasibly be retrievable from the annual guava yield losses. This is because the quantity of marketable yield (112 kg ha^{-1}) to repay cost of spot treatments is just about 5.71% of the annual guava yield losses (1,963 kg ha^{-1}), whereas the full amount of yield (2,063 kg ha^{-1}) required to repay costs of applying *Heterorhabditis bacteriophora* by hectare-wide EPN-augmentation method cannot feasibly be recovered from the annual yield losses. This is because the amount of losses is smaller than the guava yield needed to repay cost of hectare-wide EPN-augmentation.

Discussion

The relationships observed between EPN efficacy and increasing IJ rate of *Heterorhabditis bacteriophora* in the field were similar to the type of relationship between increasing IJ rate of *Heterorhabditis bacteriophora* and the mortalities of Caribfly larvae we observed in our recent study under laboratory conditions (Heve et al., 2017a). In numerous reports,

similar relationships have been observed between mortalities of other fruit flies and increasing IJ rate of *Heterorhabditis bacteriophora*, *S. carpocapsae*, *H. baujardi* or unknown species of *Heterorhabditis* following laboratory trials (Toledo et al., 2006; Rohde et al., 2012; Minas et al., 2016). Treatments to two infested fruit with *Heterorhabditis bacteriophora* at 100 IJs cm^{-2}, equivalent to 17,500 IJs $fruit^{-1}$, were the most promising and achieved maximum control of *Anastrepha suspensa* in the field. The negative effects of intraspecific competition or interference, at higher IJ rates on the efficacy, that we observed in our laboratory trials also occurred in the field here with reduced efficacies as IJ rates increased beyond the effective IJ rates at which maximum or optimum control of Caribfly was observed in plots (Heve et al., 2017a).

In our previous report, multiple EPN species treatments to five Caribfly-infested guava fruit at 352 IJs cm^{-2} did not significantly reduce the densities of emerging parasitoid wasps (*Diachasmimorpha longicaudata*) in the field (Heve et al., 2017b, 2018). The current EPN-dose response study revealed high probability (40-85%) to observe declining densities of emerging parasitoid wasps as IJ rate increases, thereby supporting the small negative value of the insignificant Pearson correlation coefficient ($r = -0.166$; $P = 0.307$) observed for the linear relationship between relative survival of the parasitoid wasps (*Diachasmimorpha longicaudata*) and increasing *Heterorhabditis bacteriophora* IJ rate from 0 to 100 IJs cm^{-2}. Nonetheless, accumulated numbers of parasitoid wasps that emerged marginally decreased with increasing IJ rate (0-100 IJs cm^{-2}), indicating that some surviving wasps (*Diachasmimorpha longicaudata*) will still be observed when *Heterorhabditis bacteriophora* is used for suppression of Caribfly in orchards (Heve et al., 2017b, 2018). Perhaps, Caribfly may be better managed using *Heterorhabditis bacteriophora* at the best or optimum IJ rate with the release of *Diachasmimorpha longicaudata* than using the EPN species alone, because both effectively attack tephritid pests (Sivinski et al., 1996; Montoya et al., 2000; Vargas et al., 2012; Weems et al., 2014; Schliserman et al., 2016; Simmonds et al., 2016; Heve et al., 2017a, b, 2018). However, future studies involving releases of large numbers of the parasitoid wasps (*Diachasmimorpha longicaudata*) in the field are required to examine the effects of EPNs on relative survival of the parasitoid *Diachasmimorpha longicaudata* in EPN treatments. This is because only a few emerging wasps (*Diachasmimorpha longicaudata*) were observed in the field.

When five fruit were periodically added to plots and then treated with 2.8 times as many *Heterorhabditis bacteriophora* IJs $fruit^{-1}$ as the best rate in this study for five times in a year, 80% suppression of Caribfly was achieved (Heve et al., 2017b, 2018). But in the current study, similar EPN treatments with *Heterorhabditis bacteriophora* to two fruit added to plots for two consecutive times caused 60% reduction in densities of Caribfly at the best IJ rate. Repeating EPN treatments to fruit fly pests several times in the field normally increases EPN efficacies better than the first few EPN treatments in plots, because of accumulation of EPNs in soil following serial or intermittent EPN-augmentations (Minas et al., 2016). Possibly, the variation in the observed efficacies of *Heterorhabditis bacteriophora* may depend in part on the number of guava fruit treated at a specified IJ rate at a time, the number of times a particular IJ rate has serially or intermittently been applied in plots and the parts of the year Caribfly-infested guavas are treated with EPN in the field. EPN-augmentation strategies involving switching between different IJ rates from time to time may improve the overall suppression of Caribfly in infested guava orchards. Nonetheless, we observed in this study that an increase in the IJ rates beyond the best rate (100 IJs cm^{-2} or 17,500 IJs $fruit^{-1}$) mostly decreased efficacies of *Heterorhabditis bacteriophora*.

Rotten guavas, the majority being infested by Caribfly, are often observed on the ground across orchards in south Florida and these guavas largely constitute the annual yield losses (Crane and Balerdi, 2016). The losses are *ca*.18% of total annual guava yield (Evans et al., 2018) and may be valued at about US$ 8,650 ha^{-1} if they are sold at the current base price of US$ 4.41 kg^{-1}. Since only small amount (i.e., about 112 kg ha^{-1}) of saleable ripening guavas will have to be recovered from the annual yield losses in order to repay the costs of EPN-augmentation by spot treatments, we considered application of *Heterorhabditis bacteriophora* over individual fruit in spots from March to July to be more feasible than hectare-wide EPN-augmentation (or broadcasting) strategy. Suspensions of active *Heterorhabditis bacteriophora* IJs at the rate 17,500 IJs $fruit^{-1}$ can be measured or calibrated in small volumes of tap-water and then spread over each fruit on the ground, using any equipment that can release and spread the required amount of IJ suspension over each fruit on soil surface. However, future study should examine the impact of *Heterorhabditis bacteriophora*-augmentation by spot treatment in Caribfly IPM plans on recovery of uninfested ripening guava yield from the annual yield losses.

The insecticides, spinosad-based product (i.e., GF120™) and malathion, are currently used to manage Caribfly in south Florida because the insecticide method appears to be the least costly among the available Caribfly management strategies. But guava growers occasionally combine pesticide use with other Caribfly man-

agement practices involving citronella mixture which is a fruit fly repellent, the removal or destruction of fruit on the ground and trapping a few adult flies on sticky materials (personal communications with growers). These combinations tend to better reduce the Caribfly infestation of the saleable high flavor 'light green-to-yellow' ripe guava fruit than using any of these methods alone. Nevertheless, high costs involved in combining these practices against Caribflies tend to control sustainability. Bagging guava fruit on trees is the only method that prevents 100% of infestations of ripening guavas in Caribfly-infested orchards. But the current costs of bagging fruit for higher wholesale prices mainly in organic guava markets are high, i.e., US$ 0.29 fruit^{-1}. Currently, most of the guava growers largely harvest the non-susceptible mature hard 'dark green' guava fruit to escape infestation of fruit by Caribfly. However, some amounts of marketable 'light green-to-yellow' ripe guavas are still lost due largely to Caribfly infestation of exposed or unbagged guava fruit which ripen on guava trees to become nearly yellow in appearance with an increase in size and flavor (Crane and Balerdi, 2016). This requires the need for the growers to adopt a comprehensive Caribfly IPM to effectively reduce the Caribfly infestation rates.

Heterorhabditis bacteriophora appears to be more promising to control fruit-to-soil life cycle stages of Caribfly (Heve et al., 2017a, b, 2018). Our profitability analysis showed that guava growers can better afford combining costs of *Heterorhabditis bacteriophora* spot treatments and those of insecticides in Caribfly IPM to control Caribfly 'fruit-to-soil' stages belowground and adult Caribfly aboveground, respectively. However, the commercial prices of *Heterorhabditis bacteriophora* and those of similar virulent EPN species such as *H. indica*, *S. feltiae*, *S. carpocapsae*, *S. riobrave*, and *S. glaseri*, among others, may be considered a major factor to minimize costs. In our previous report, the EPN species *H. indica*, *S. feltiae*, and *S. carpocapsae* were more virulent to Caribfly larvae than they were to pupae, whereas *S. glaseri* and *S. riobrave* better suppressed emerging adult Caribfly from the resistant Caribfly pupae buried in soil microcosms even though *S. riobrave* killed fewer Caribfly larvae than *S. glaseri* did (Heve et al., 2017a). In our recent field trials, *H. indica* (the native species in Florida), *S. feltiae* (exotic species), and *Heterorhabditis bacteriophora* (also an exotic) suppressed emerging adult Caribfly from Caribfly-infested guava fruit in a similar manner (Heve et al., 2017b, 2018). These suggest that the virulent nematodes *H. indica*, *S. feltiae*, *S. carpocapsae*, *S. riobrave*, or *S. glaseri* may be preferred in Caribfly IPM to *Heterorhabditis bacteriophora* which effectively attacks all the fruit-to-soil stages of Caribfly better (Heve et al., 2017a, b, 2018). But observations by Heve et al. (2017b, 2018) suggest that application of mixtures of these EPN species may reduce efficacy against Caribfly. Guava growers should adopt the use of EPN together with insecticides across all guava orchards in south Florida so that the control of the flies can be more effective. Nevertheless, the information and scientific procedures in this study may be useful and applicable in many similar situations where damaging insect or tephritid pests are problems on food crops such as fruit and vegetables.

Acknowledgments

Appreciation to CREC-UF/IFAS (Lake Alfred FL), TREC-UF/IFAS (Homestead FL), USDA (Miami FL), FDACS (Gainesville FL), Department of Entomology and Nematology of University of Florida, and the Tropical Lacosta Inc. for support to the first author (W. K. Heve) during his PhD research work in south Florida.

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