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Arthropod Community Responses Reveal Potential Predators and Prey of Entomopathogenic Nematodes in a Citrus Orchard

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Abstract: The contributions of soil arthropods to entomopathogenic nematode (EPN) food webs are mainly studied in artificial conditions. We investigated changes in arthropod communities in a citrus orchard following soil inundation with *Steinernema feltiae* or *Heterorhabditis bacteriophora*. We hypothesized that arthropod taxa, which decline or increase in response to EPN augmentation, represent potential prey or predators of EPN, respectively. Soil was sampled periodically after nematodes were applied, DNA was extracted from organisms recovered by sucrose centrifugation, libraries were prepared, and the ITS2 and CO1 genes were sequenced using Illumina protocol. Species from 107 microarthropod (mites and collembola) families and 121 insect families were identified. Amplicon sequence variant (ASV) reads for *H. bacteriophora* were less than 10% of those for *S. feltiae* three days after inundation, whereas microarthropod ASVs were double in plots with *H. bacteriophora* compared to those with *S. feltiae*. Significantly fewer microarthropod and insect reads in *S. feltiae* compared to untreated plots suggest the possibility that *S. feltiae* preyed on mites and Collembola in addition to insects. The responses over time of the individual microarthropod species (MOTU) suggest that regulation (up or down) of these EPN resulted from a cumulative response by many species, rather than by a few key species.

Keywords: entomopathogenic nematodes; soil microarthropods: food web response; metabarcoding; nematodes augmentation

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1. Introduction

Entomopathogenic nematodes (EPNs) belong to two families, Steinernematidae and Heterorhabditidae, both with a worldwide distribution except for Antarctica. First noted a century ago [1], EPNs received little attention for several decades until their potential for biological control became increasingly apparent [2]. Now they are among the most well-studied soil taxa, both for insect management and as model systems for symbioses such as parasitism and mutualism [3]. All steinernematid and heterorhabditid species are obligately associated with bacterial species in the genera *Xenorhabdus* or *Photorhabdus*, respectively. The bacteria are also entomopathogens that are released into the insect hemocoel after the nematodes gain entry either through natural body openings or by penetrating the cuticle. Insects die from septicemia and the nematodes, and bacteria consume the tissue while completing several generations. Nematode development arrests at the 3rd stage when conditions in the cadaver deteriorate, and a cohort of tens of thousands of infective juveniles (IJs) emerges in search of new hosts. Several of the more than 100 described EPN species are currently formulated as inundative biological insecticides that are applied to soil or foliage to help manage a growing number of insect pest species [4]. Exotic EPN species have been employed successfully in classical biocontrol programs targeting invasive pest insects [5]. Conservation biocontrol tactics to better exploit EPN services have also been studied and proposed [6,7].

Conserving or enhancing biocontrol by augmented [8,9] or indigenous [10,11] EPNs would be desirable, given the high cost of producing them and the generally low persistence of most species following release. Smits [12] and Griffin [13] described a five-phase EPN inoculum progression model that exhibits a rapid, major decline immediately upon application, followed by further gradual decline and eventually a period of maintenance (at or below detectable levels) or local extinction. Physical forces affecting EPN survival [14–16] and efficacy [17,18] are recognized to some extent, but less is known about how food webs affect the post-application survival of commercial EPN [3]. While abiotic stressors of EPN, such as UV radiation and desiccation, are lethal initially, a myriad of EPN antagonists inhabit the surface and deeper soil horizons. Indeed, most common guilds of soil-dwelling organisms are thought to be capable of modulating EPN populations in some manner [13]. Nevertheless, the extent to which specific taxa drive measurable predator–prey dynamics is virtually unknown [19]. Plentiful laboratory assays have identified microarthropods—mites, collembola, monurans and diplurans—that readily graze and persist on nematodes [20–23]. Rarer are studies of microarthropod numeric responses to EPN in nature [24–26]. The roles of microarthropods [27] and non-pest insects [28] as hosts that support EPN persistence are virtually unknown. Nematophagous fungi and their associations with EPN have been subjects of numerous laboratory experiments [29,30] and field studies [31,32]. Bacterial ectoparasites [33,34] and free-living nematodes that scavenge within insect cadavers appear to be major, widespread EPN antagonists [25,35–37].

The capacity to study suites of diverse, cryptic organisms in nature expanded significantly with advances in qPCR technology [38,39], and the continued development and cost reduction of metagenomic tools now provide the potential to detect all species in an environmental sample for which a given gene region is represented in the molecular databases [40]. The enormous expansion in the breadth of taxonomic coverage afforded by metabarcoding of environmental DNA increases the opportunity to detect species that interact directly with EPN or are measurably affected by them indirectly. The technology also broadens the scope of possible inquiry. For example, comprehensive coverage of the soil community responses to EPN augmentation could reveal whether characteristics of some EPN species or genera make them broadly more susceptible to predation (less likely to persist) or more rhizosphere competent as efficient predators of diverse organisms.

Here, we describe changes in an orchard soil community as measured by metabarcoding of soil DNA following augmentation with two EPN species. Our objective was to identify potential antagonists and prey of EPN based on significant differences between populations in augmented and non-augmented plots. We used universal primers to build libraries from ITS2 rDNA and COI mtDNA to target arthropods and nematodes. We hypothesized that some predators or competitors of EPN would increase in abundance, whereas specific prey of EPN would measurably decrease following appreciable EPN augmentation. Therefore, significant differences between populations in different treatments should represent potential predators, prey, competitors or indirectly affected nontargets. We anticipated different food web responses to the two augmented species because we expected a more rapid decline in the numbers of amplified sequence variants (ASV) of *Heterorhabditis bacteriophora* (Poinar, 1976), compared to *Steinernema feltiae* (Filipjev, 1934) [41]. Compared to the control plots, we predicted an overall reduction in the number of insect (prey) ASV and an increase in those for microarthropods (predators) in the EPN-augmented plots. We also anticipated responses, both positive [42] and negative [43], by some nematode species.

2. Materials and Methods

The experiment was conducted on a 0.1 ha plot in a mature citrus orchard in Polk County, central Florida (28.240771083739954, −81.76575482177549). The experimental design was randomized complete block with 8 replications of three treatments, *H. bacteriophora* (Nemasys®, BASF Corportation), *S. feltiae* (Nemasys G®, BASF Corportation), and an untreated control. The experimental units were single citrus trees (24 trees in total). Entomopathogenic nematodes were applied to a square area two meters per side under the tree

canopy, by spaying the soil surface with 300 infective juveniles per square centimeter (12 M IJs per tree). Compressed nitrogen was used to spray aliquots of nematodes suspended in 10 L water at 1.41 kg/cm^2 pressure. Microjet irrigation occurred during the application and for one hour afterward.

Soil samples were taken from all 24 trees at 3, 7, 14, and 28 days after nematode application. A soil sampling tube was used to collect eight soil cores (dia 2.5 cm × 30 cm depth) from each plot, which were combined into a single composite sample of approximately 2000 mL volume. Each composite sample was gently mixed and then nematodes, soil microarthropods, insects and associated microorganisms were extracted by sucrose centrifugation from 250 cm^3 of soil [44] and collected in falcon tubes of 15 mL. After aspirating excess water, soil samples were concentrated in Eppendorf tubes where DNA was extracted with DNeasy® PowerSoil Kit (Qiagen). The DNA concentrations were measured using the Qubit® dsDNA High-Sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Two libraries were created because two set of primers were employed to target two groups—nematodes and soil microarthropods. For the nematodes, the target region was ITS 2 of the rDNA gene using previously reported primers. The average amplicon length was 450 bp for steinernematids and 350 bp for heterorhabditids. The universal primers were AD58F (5′-TCGATGAAAAACGCGGCAA-3′; [40]) forward and AB28R (5′-ATATGCTTAAGTTCAGCGGGT-3′; [45]) reverse. For microarthropods, universal primers for COI of mitochondrial DNA were mlCOIintF (5′- GGWACWGGWTGAACWGTWTAYCCYCC; [46]) forward, and jgHCO2198 (5′- TAIACYTCIGGRTGICCRAARAAYCA-3′; [47]) reverse.

Following the Illumina protocols, library preparation consisted of two amplifications and two clean up steps. For the first amplification "amplicon PCR", overhang adapter sequences were appended to the primer pair for compatibility with Illumina index and sequencing adapters. Samples were standardized at 5 ng/mL DNA concentration. For nematodes, samples were amplified with the following conditions: initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 98 °C for 20 s, annealing at 56.5 °C for 30 s, elongation at 72 °C for 60 s and terminal elongation at 72 °C for 10 min. The same PCR conditions were used for microarthropod amplification, with the only difference of an annealing temperature at 50 °C for 30 s. For all libraries, a single 25 µL PCR reaction containing 2.5 µL of template at 5 ng/µL (12.5 ng total), 12.5 µL of NEBNext® High-Fidelity 2X PCR Master Mix (New England biolabs), 1 µL of each 10 pM overhang primer and 8 µL of 10 mM Tris pH 8.5 was used. DNA extracted from a laboratory culture of the nematodes *Steinernema glaseri* (Steiner, 1929) and *Heterorhabditis indica* (Poinar, Karunakar & David, 1992) were used as positive controls, while negative controls consisted of purified, nuclease-free water instead of template.

PCR products were verified on 1% agarose gels after staining with SYBR™ Safe DNA Gel Stain. All amplicon PCR products were purified with 1.0 × HighPrep™ PCR post PCR clean up system (MagBio Genomics, Inc., Gaithersburg, MD, USA) and eluted in 50 µL of 10 mM Tris pH 8.5. For the following index PCR, amplicons were used as template for a limited cycle amplification adding dual-index barcodes: P5 and P7 Illumina sequencing adapters using Nextera XT Index Kit (FC-131-1001) for EPN and XT Index Kit (FC-131-1004) for microarthropods (Illumina, San Diego, CA, USA). The index PCR conditions were initial denaturation at 95 °C for 3 min, 8 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s and a terminal elongation at 72 °C for 10 min. Each 50 µL PCR reaction tube contained 5 µL of template, 25 µL of 2× KAPA HiFi HotStart ReadyMix (KAPA biosystems), 5 µL of Index Primers (N7XX) and 5 µL of Index 2 Primers (S5XX). The total number of 192 index PCR products purified with 1.1× magnetic beads, eluted in 25 µL and quantified using Qubit 3.0 fluorometer. Finally, libraries were normalized in equal molar concentrations of 4 nM and pooled together in a single library in aliquots of 10 µL. The library was sequenced using MiSeq 2 × 300 bp paired-end Illumina at the Interdisciplinary Center for Biotechnology Research (ICBR) of University of Florida.

Bioinformatics: ICBR delivered raw data in fastq format, which were demultiplexed and separated into respective sample identification codes. FASTQC v0.11 (Andrews et al., 2015) was used for quality assessment of each read, and then all the quality information was combined into a single viewable document using MULTIQC (Ewels et al., 2016). The constructed libraries of ITS 2 rDNA and COI mtDNA were used for nematode and microarthropod identification, respectively. In both datasets, R1 and R2 reads were combined and dereplicated with the ASV-based approach, in which DADA2 was the denoising method, through the QIIME2 v2019.4 pipeline, including removal of primer sequences, truncating sequences by length and removing chimeric sequences with a de novo approach according to Callahan [48], which resulted in a length of 350–450 bp for nematodes and 313 bp for microarthropods. We then generated count tables by mapping ASVs, assigning taxonomy by generating input files for taxonomy assignment in QIIME2 from the NCBI database. A standalone database was generated including all the non-redundant nucleotide sequences from all traditional divisions of GenBank (ftp://ftp.ncbi.nlm.nih.gov/blast/db/nr.gz; accessed on 20 December 2020.) employing an NCBI command-line tool (BLAST+) to integrate BLAST directly into our workflow. BLAST taxonomy was assigned to ASVs to create molecular operational taxonomic units (MOTUs) defined by the NCBI accession numbers. For statistical analyses, MOTU datasets were corrected according to the sample DNA quantity and then were log transformed. Analysis of variance was performed primarily on all three major groups (total sum of the reads) and the MOTUs within the groups (389 Microathropod MOTUs, 311 insect MOTUs and 20 nematodes species) applying a repeated measures approach (time, plot and treatment) where experimental plots (trees) were randomly nested within the three treatments. For each significant taxa, post hoc analysis was performed to determine differences between the means using Least Significant Difference (LSD-test) at which *p*-values were adjusted by a Bonferroni correction approach. To eliminate false positives, we analyzed only taxa that were detected in 75% or more of the sites. All statistical datasets were created in R (R Development Core Team). The 'agricolae' package [49] was used for the statistical analyses, and plots were reproduced by 'Sigmaplot' version 14.0 and 'R studio'. Treatments' effects on native nematodes and microarthropods were further explored with redundancy analysis using Canoco v.5 [50].

3. Results

The high-throughput sequencing produced two datasets, based on the two DNA loci. The ITS2 revealed 4,800,700 reads of which 51.7% (2,485,154) passed the quality filters and were denoised, merged and characterized as non-chimeric. Dereplication resulted in 6297 unique amplicon sequence variants (ASVs), of which 44% (2793 ASVs) were characterized as unidentified and 4.7% (301 ASVs) as Nematoda. Combining ASVs with the same accession number, removing all ASVs with less than 10 reads and setting a threshold of 75% coverage, we identified 18 species of nematodes that were the basis of further statistical analyses. The COI dataset yielded 7,060,355 reads that reduced to 5,691,737 (80.6%) after filtering, denoising, merging and chimera removal. Dereplication resulted in 6702 ASVs, of which 1.3% (92 ASVs) were characterized as unidentified, and by using the same restrictions as for ITS2, we produced 398 MOTUs of soil microarthropods from 108 families (93 mites, 13 collembolans and 2 diplurans) and 307 insect MOTUs from 122 families. All identified nematode, microarthropod and insect species are given in Supplementary Table S1.

Augmented EPN species differed in persistence. Three days following augmentation, an average of 7136 *H. bacteriophora* reads compared to 91,180 *S. feltiae* reads were detected in their respective plots. Between days 3 and 28, *H. bacteriophora* reads declined an additional 54% (3285) compared to 36% for *S. feltiae* (57,998) (Table 1).

Both nematode treatments increased the total nematode read abundance (Figure 1, top left panel). There were significant treatment–time interactions for both the total number of nematode reads ($p = 0.002$; adjusted $R^2 = 50.4$; Supplementary Table S2) and those of the native nematodes ($p = 0.002$; adjusted $R^2 = 35.5$; Supplementary Table S3): the addition of *S. feltiae* increased the read abundance of identified nematodes above that in the control

plots for at least two weeks, whereas *H. bacteriophora* had no significant effects on the total nematode reads at any time (Figure 1, top right panel). The numbers of native nematode reads responded to EPN augmentation, declining below the levels in untreated plots on day 7 in plots amended with both species and on day 28 in plots amended with *S. feltiae* (Figure 1, bottom right panel; Supplementary Figure S1). Although the observed reads were fewer ($p < 0.001$) in plots treated with *S. feltiae* (46526 ± 7694) than those augmented with *H. bacteriophora* (96383 ± 27201), neither treatment differed significantly from the control (64400 ± 8400) (Figure 1, bottom left panel).

Table 1. Abundance of *Steinernema feltiae* (Sf) and *Heterorhabditis bacteriophora* (Hb) for 28 days following augmentation in a citrus orchard.

	Hb		Sf	
Sampling Day	**Mean**	**SE**	**Mean**	**SE**
3rd	7135.82	2500.1	91,180.1	29,235.3
7th	6085.63	1373.1	76,452.6	31,251.9
14th	2244.35	616	81,742.2	49,325.8
28th	3285.4	1373.5	57,997.7	26,907.1

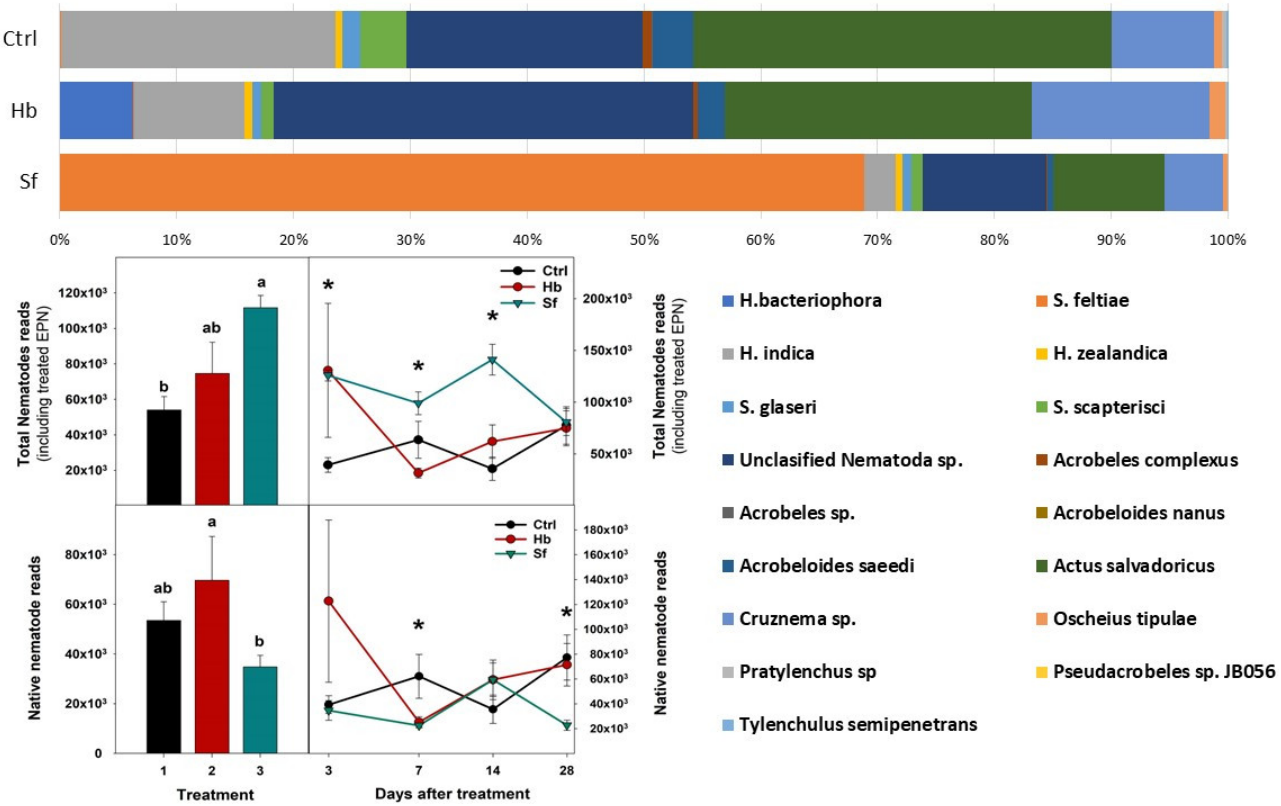

Figure 1. Results of DNA metabarcoding of nematode communities. **Upper panel:** Stacked bar plots showing the relative abundance of nematode species (**listed lower right**) detected in a citrus orchard for 28 days following augmentation with *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb) and an untreated control (Ctrl). Asterisks (*) indicate significant differences ($p < 0.05$) between the treatments. **Lower left:** Two bar plots of the nematode read abundance in each treatment: upper bar plot is of total nematode reads (augmented plus native species) and lower bar plot depicts reads of native species only. **Lower center:** Temporal trends in nematode read abundance in the treatments for 28 days following augmentation. Error bars are standard errors of the mean, bars with the same letters do not differ ($p < 0.05$).

Four indigenous entomopathogenic nematodes were detected at relatively high frequency over time in the 24 sites, with *Heterorhabditis indica* (Poinar, Karunakar & David, 1992) occurring in 100% of the trees and 87.5% of the total samples, along with *Heterorhabditis zealandica* (Poinar, 1990) in 25%/15.6%, *Steinernema glaseri* (Steiner, 1929) in 83.3%/61.4% and *Steinernema scapterisci* (Nguyen & Smart, 1990) in 79.1%/42.7%, respectively. The identities were confirmed by phylogenetic analysis and, in some cases, by using species-specific qPCR primers/probes. The only nematode species with detectible responses to the treatments were bacterivores *Acrobeles complexus* (Thorne, 1925) ($p < 0.003$) and *Acrobeloides saaedi* (Siddiqi, De Ley and Khan, 1992) ($p < 0.065$) and a plant parasitic nematode *Pratylenchus* sp. ($p < 0.068$) (Figure 2). There were several positive associations between native EPN and bactivorous nematodes, including *S. glaseri* and *Acrobeles* sp. (Spearman's $\rho = 0.30$, $p = 0.003$), *H. indica* and *A. complexus* ($\rho = 0.41$, $p = 0.0001$), *H. indica* and *A. saeedi* ($\rho = 0.23$, $p = 0.02$), *Oscheius tipulae* (Lam & Webster, 1971) and *A. complexus* ($\rho = 0.23$, $p = 0.03$) and *Oscheius tipulae* and *Pseudoacrobeloides* sp. ($\rho = 0.29$, $p = 0.005$).

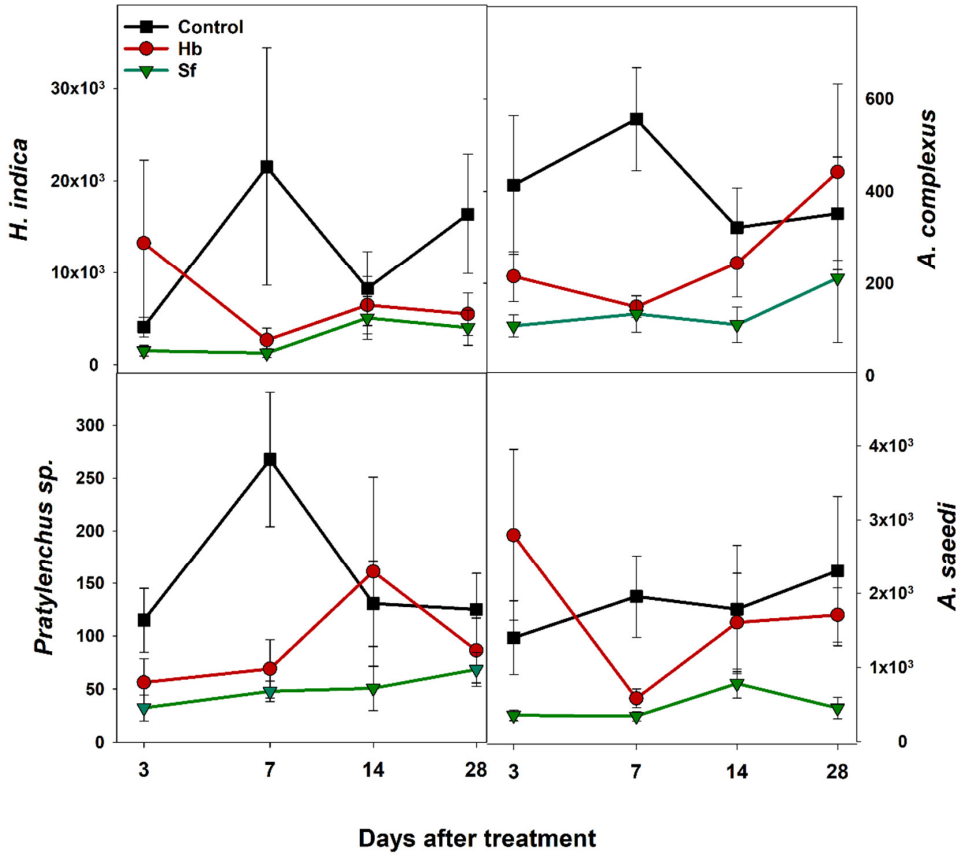

Figure 2. Read abundance of four indigenous nematode species in a citrus orchard for 28 days following augmentation of *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb) or no augmentation (Ctrl). Error bars are SE of the means.

The COI primers revealed 10 orders and 107 families of soil microarthropods comprising mites, collembolans, proturans and diplurans. As a group, microarthropods responded differently to the two inoculated EPN species. Microarthropod abundance and richness were greater in plots treated with *H. bacteriophora* than in those with *S. feltiae* (Figure 3, Table 2; Supplementary Figure S2), while neither treatment differed from the control. This pattern was mostly driven by the mites, which comprised 80% of microarthropod reads (Figures 3 and 4). The *S. feltiae* treatment also reduced collembolans with respect to both *H. bacteriophora* and control, while no treatment differed in Collembola richness (Table 2).

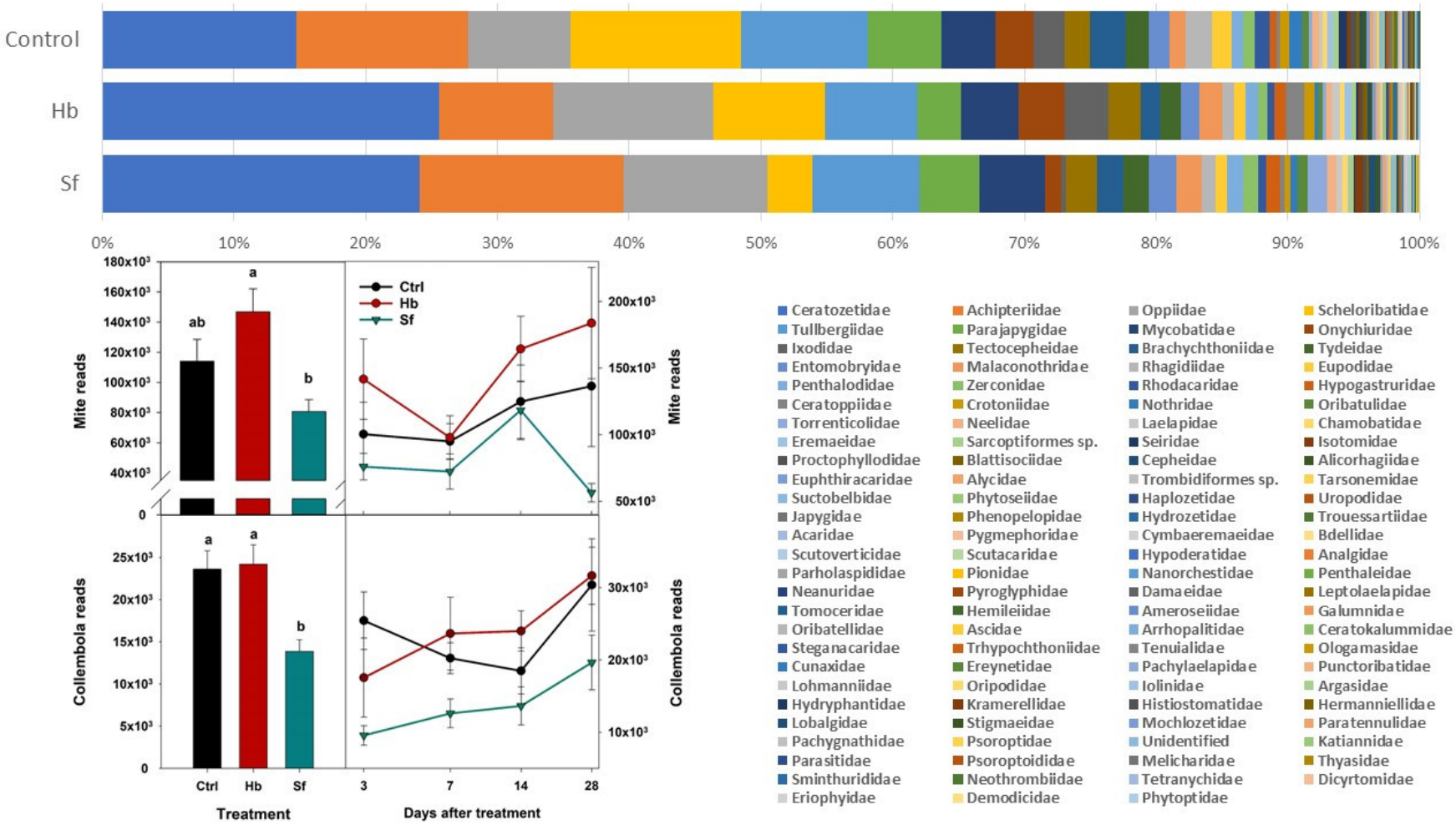

Figure 3. Results of DNA metabarcoding of soil microarthropod communities. **Upper panel:** Stacked bar plots showing the relative abundance of microarthropod families (**listed lower right**) detected in a citrus orchard for 28 days following augmentation with *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb) and an untreated control (Ctrl). **Lower left:** Bar plot of two dominant groups acari mites and springtails read abundance in each treatment. **Lower center:** Temporal trends in acari mites and springtails read abundance in the treatments for 28 days following augmentation. Error bars are standard errors of the mean, bars with the same letters do not differ ($p < 0.05$).

Table 2. Motu richness (*R*) of major taxa in the plots augmented with *Heterorhabditis bacteriophora* (Hb), *Steinernema feltiae* (Sf) and the untreated controls (Ctrl). Means in rows with the same letters do not differ ($p < 0.05$).

	Ctrl			Hb			Sf		
Group	**Mean**	**Std Error**		**Mean**	**Std Error**		**Mean**	**Std Error**	
Microarthropods	65.19	1.81	ab	65.53	1.98	a	59.63	1.88	b
Mites	53.88	1.74	ab	55.34	1.84	a	49.44	1.58	b
Collembola	5.75	0.25	a	5.44	0.22	a	5.31	0.24	a
Insects	32.59	1.07	a	30.44	1.20	ab	26.78	0.87	b
Nematodes	8.94	0.20	a	8.53	0.27	ab	7.94	0.22	b

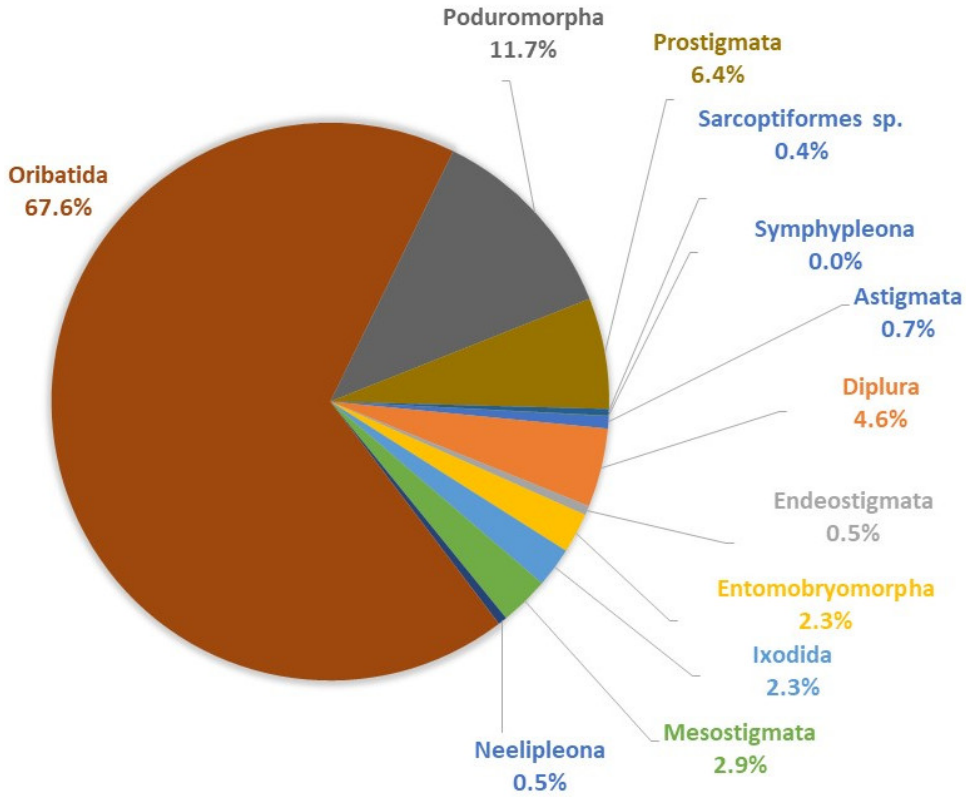

Figure 4. Pie chart of proportional composition of major microarthropod groups recovered from high-throughput sequencing of DNA recovered from soil in a citrus orchard for 28 days following augmentation of two EPN species or no augmentation.

Eight microarthropod families responded ($p < 0.10$) to the treatments with altered population dynamics. Ceratozetidae (Acari: Oribatida), Onychiuridae (Collembola: Poduromorpha), Tectocepheidae (Acari: Oribatida) and Laelapidae (Acari: Mesostigmata) exhibited population flux that was potentially driven by taxa that prey on EPN, whereas patterns for Tullbergiidae (Collembola: Poduromorpha), Japygidae (Diplura) and Eupodidae (Acari: Prostigmata) suggest the possibility that some taxa may serve as hosts that sustain EPN (Figure 5). Predictably, the population patterns of taxa within families were inconsistent (not shown), and just seven microarthropod MOTUs from the Oppiidae (Acari: Oribatida), Eupodidae (Acari: Prostigmata) and Tydeidae (Acari: Prostigmata) exhibited significant response to the treatments (Table 3). Two taxa increased in response to *H. bacteriophora* augmentation, while four species in *S. feltiae* plots declined. The difference in number of reads between those species that responded significantly to *H. bacteriophora* and *S. feltiae* was just 2.1% as numerous as the difference in the reads among the remaining species.

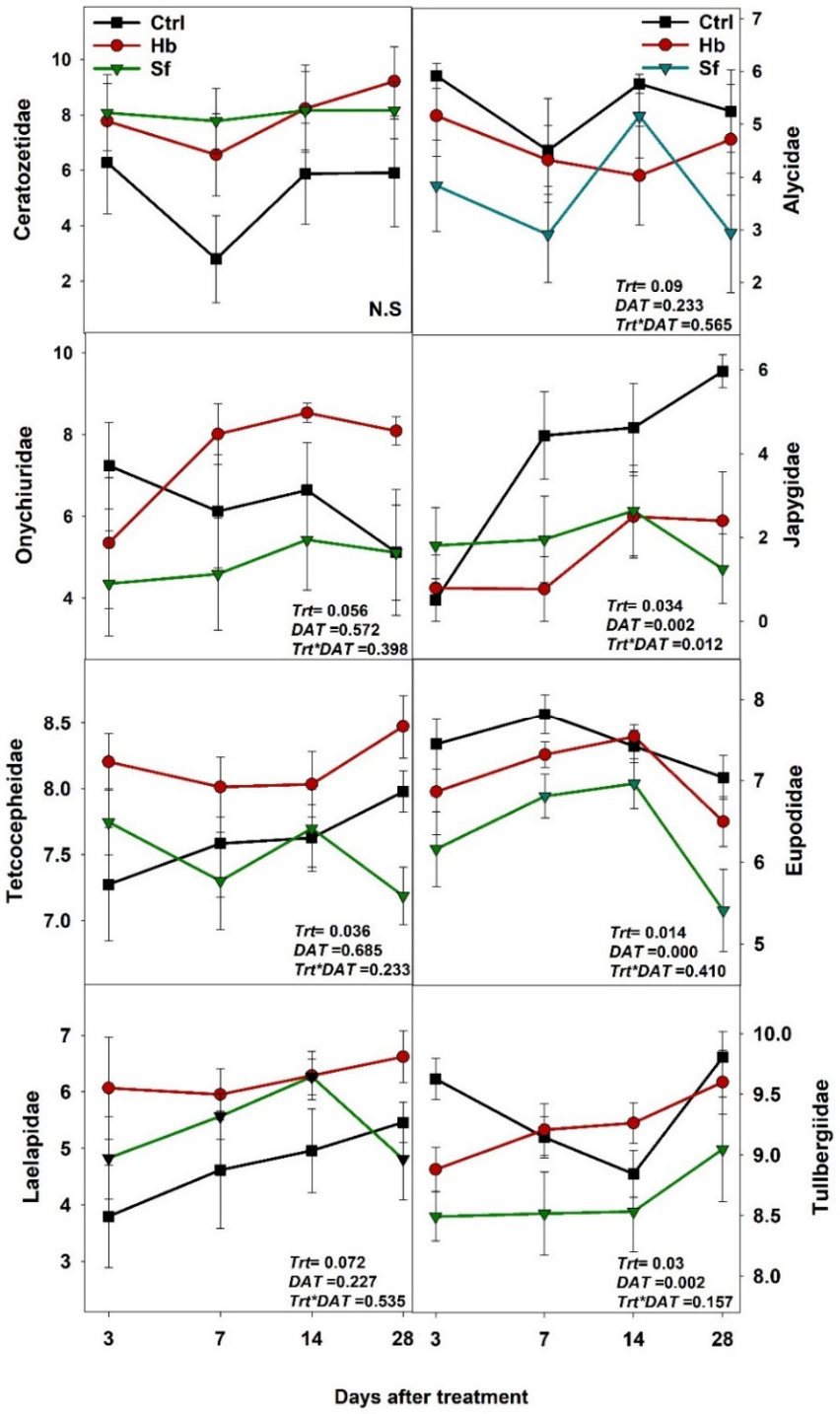

Figure 5. Eight microarthropod family responses to augmentation with *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb) and an untreated control (Ctrl). Plots in the left column exhibit potential predator epidemiology. Plots in the right column depict potential prey of entomopathogenic nematode species.

Augmented *S. feltiae* reduced the abundance of the ant *Myrmica semiparasitica*, whereas a geometrid moth *Stamnodes affiliate* increased in response to *H. bacteriophora* (Table 3). The effects of *S. feltiae* were apparent on the entire class of Insecta, which maintained population density throughout the trial in untreated plots but declined markedly in response to the steinernematid to about one-third of control levels by the end of the trial (Figure 6).

Table 3. Significant responses of MOTUs in the four major groups (microarthropods, insects and nematodes) according to two-way repeated measures ANOVA. R squared values show proportion of variance that can be explained by the independent variable. Means comparison performed using Least Significant Difference (LSD-test) with *p*-values adjusted by the Bonferroni correction approach. Treatments with the same letter following mean read abundance of the untreated control (Ctrl), *Heterorhabditis bacteriophora* (Hb) and *Steinernema feltiae* (Sf) do not differ ($p < 0.05$). Abundance indicates the total read abundance of the family. Analysis was applied only to taxa occurring in more than 75% of the 24 treated plots (Plot freq), while "Total freq" indicates the frequency of occurrence in the 96 samples.

Accession	R^2/R^2(adj)	Treatment	Sampling Day	Interaction	Ctrl		Hb		Sf		Description	Family	Abundance	Freq in Trees	Total Freq
Microarthropods															
JX836034.1	0.396/0.316	0.011	0.688	0.740	348.30	b	910.85	a	89.35	b	Oppiidae sp. MYMCC093-11 voucher BIOUG01067-93	Oppiidae	43,152	0.75	0.43
MG316849.1	0.368/0.284	0.029	0.386	0.131	227.54	a	105.87	ab	50.25	b	Eupodidae sp. BIOUG25723-A11	Eupodidae	12,277	0.96	0.68
MG320390.1	0.472/0.403	0.034	0.008	0.640	783.33	ab	933.56	a	597.27	b	Tydeidae sp. BIOUG25166-E03	Tydeidae	74,053	1.00	0.89
MN349530.1	0.282/0.188	0.034	0.150	0.292	525.53	a	721.48	a	304.87	b	Eupodidae sp. BIOUG23551-E05	Eupodidae	49,660	1.00	0.96
MG321080.1	0.323/234	0.046	0.095	0.821	34.77	a	20.82	ab	12.66	b	Eupodidae sp. BIOUG25167-C10	Eupodidae	2184	0.75	0.36
MG317718.1	0.475/0.407	0.022	0.555	0.083	74.90	ab	98.40	a	10.90	b	Tydeidae sp. BIOUG26106-F03	Tydeidae	6851	0.71	0.33
Insects															
GQ255183.1	0.472/0.403	0.017	0.576	0.244	6417.96	a	4665.52	ab	1819.52	b	Myrmica semiparasitica voucher GJ533	Formicidae	82,665	1	0.96
HM906951.1	0.393/0.314	0.021	0.127	0.715	908.27	b	2067.97	a	871.40	ab	Stamnodes affiliata voucher BIOUG<CAN>:CCGBOLD00082	Geometridae	26,988	1	0.98
Nematodes															
NA	0.360/0.276	0.003	0.601	0.973	410.9	a	262.4	a	140.5	b	Acrobeles complexus	Cephalobidae	26,039	1.00	0.94
NA	0.648/0.601	0.065	0.564	0.038	1862.0	a	1672.2	ab	482.9	b	Acrobeloides saeedi	Cephalobidae	128,546.02	1.00	0.99

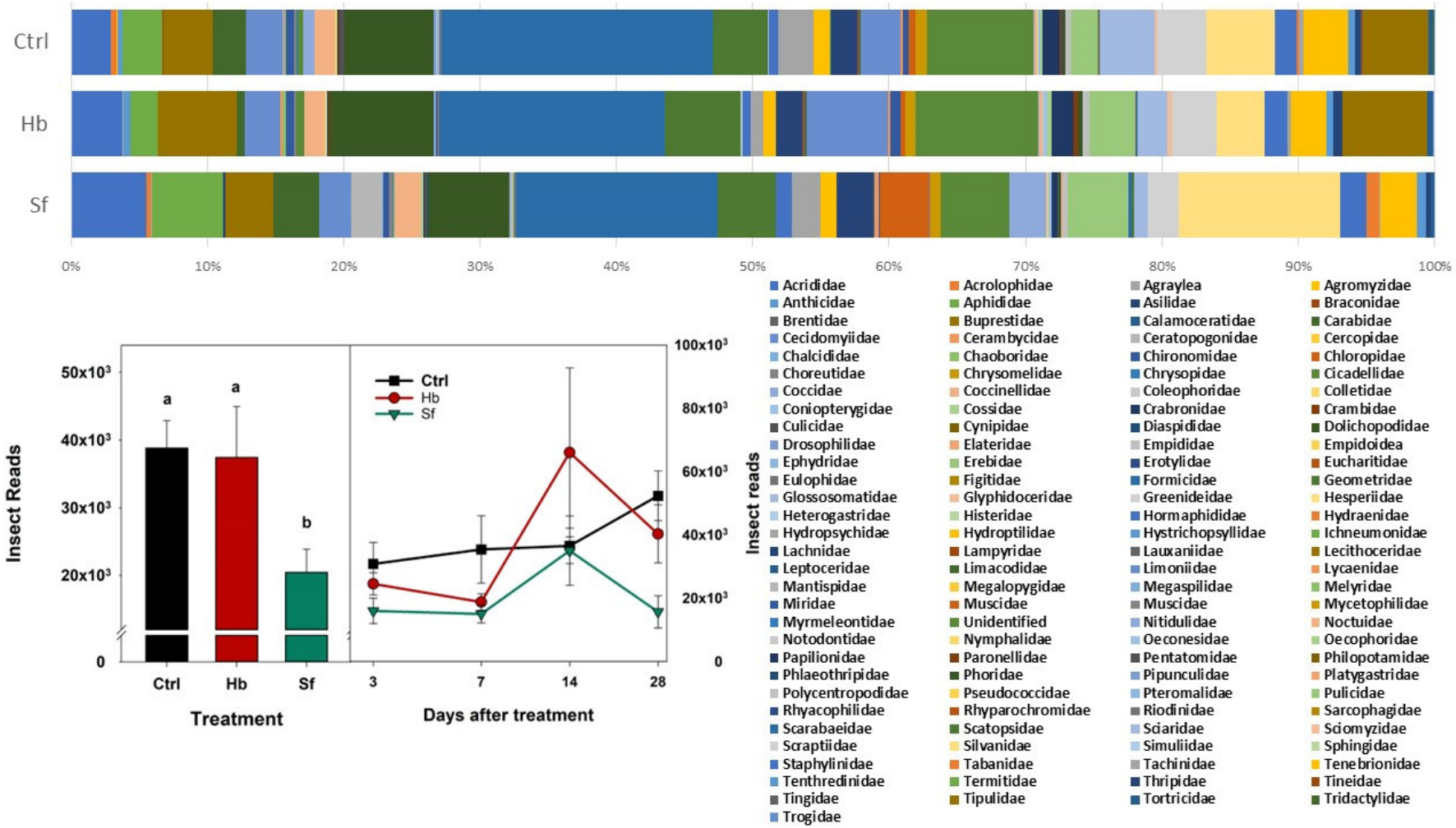

Figure 6. Results of DNA metabarcoding of insect communities. **Upper panel:** Stacked bar plots showing the relative abundance of insect families (**listed lower right**) detected in a citrus orchard for 28 days following augmentation with *Steinernema feltiae* (Sf), *Heterorhabditis bacteriophora* (Hb) and an untreated control (Ctrl). **Lower left:** Bar plot of the insect read abundance in each treatment. **Lower center:** Temporal trends in insect read abundance in the treatments for 28 days following augmentation. Error bars are standard errors of the mean and bars with the same letters do not differ ($p < 0.05$).

4. Discussion

Entomopathogenic nematode augmentation elicited responses by each of the three groups of soil organisms considered here. Compared to controls, microarthropod, insect and native nematode responses depended on the EPN species introduced into the plots. As anticipated, the post-application survival capacity of the augmented species was related to some of those patterns. Three days post-augmentation, the reads for *H. bacteriophora* were less than 10% of those for *S. feltiae*. Thereafter, the decline of both species was more gradual, but still more pronounced for *H. bacteriophora* than for *S. feltiae*. Duncan et al. [41] reported exogenous *H. bacteriophora* declining to levels that were 1% those of exogenous *S. carpocapsae* and *S. riobrave* within 5 days of application to soil beneath citrus trees. Numerous reports (reviewed by Strong [51]) indicate generally lower short-term persistence of heterorhabditids compared to steinernematids. The effect of EPN augmentation on the total abundance of nematodes and other organisms in soil can only be approximated by barcode reads because of the interspecific and life stage variability of tandem repeats [52,53]. Nevertheless, the negligible increase in the total nematode reads following application of *H. bacteriophora* compared to more than doubling in plots with *S. feltiae* was striking and suggests that the temporal impacts of EPN augmentation on food webs are highly species-specific and may be very different [26]. The total nematode reads in plots augmented with *S. feltiae* exceeded those in control and *H. bacteriophora* treatments for at least two weeks, and this was followed by reductions of native nematodes. Previous reports of EPN augmentation inducing a reduction of native EPN attributed competition or natural enemies and classical predator–prey dynamics as the likely causes [25,36,54]. Consequently, avoiding EPN augmentation immediately prior to seasonal recruitment of herbivorous larvae into soil is recommended to conserve the services of native EPN in Florida citrus [7]. Although *H. bacteriophora* inundations may have increased some soil mites early in the trial, we detected no increased natural enemy density in the *S. feltiae* treatments that might have reduced the numbers of native nematodes detected in those plots. The ability of exogenous EPN to reduce plant-parasitic nematodes has been reviewed by Kenney and Eleftherianos [55] and most plausibly attributed to allelopathy [56,57] and the induction of plant defense systems [58,59]. Plant defenses seem less likely than allelopathy to have affected the two bactivorous species shown to decrease here. Moreover, we anticipated, but did not find, an increase in some bactivorous nematode populations capable of competing with EPN for resources in insect cadavers [36,60,61]. Positive associations between EPN and bactivorous nematodes, such as those found here between bacterivores and the obligate and facultative [62] EPN *S. glaseri, H. indica* and *O. tipulae*, may require longer than 28 days to develop.

Our hypothesis that EPN augmentation would increase the total microarthropod numbers above those in controls was not supported; however, the higher microarthropod prevalence in plots treated with *H. bacteriophora* compared to *S. feltiae* may reflect higher rates of predation on the augmented heterorhabditid. Perhaps differences in rhizosphere competence of EPN species, such as those seen in this and previous studies [51], provide a more sensitive means of detecting organisms that regulate EPN as either predators or hosts. Indeed, the trends for both EPN treatments suggest not only that microarthropods preyed more on *H. bacteriophora* than *S. feltiae*, but that some mites, springtails and diplurans may have been hosts for *S. feltiae*. The feeding habits and behavior of soil mite species that prey on nematodes have been widely characterized under controlled conditions [21,22,63] and from field observations [20]. As seen in this study, Wilson and Gaugler [24] reported an inverse relationship between survival of *H. bacteriophora* and both mite and collembolan abundance in the field. Given the predatory nature of so many microarthropod species, the lack of a significant positive response by more than a single species to EPN augmentation suggested that many taxa contributed incrementally, along with unmeasured factors, to the rapid decline of *H. bacteriophora.* By contrast, there are few reports [25] consistent with the overall reduction of mites and springtails by EPN applications found here in the plots inundated with *S. feltiae.* Joharchi et al. [64] observed bulb mites *Rhizoglyphus robini*

(Acari: Acaridae) in alfalfa to consume large numbers of stem nematodes *Ditylenchus dipsaci* (Tylenchida: Anguinidae). Later, Nermut' et al. [27] described EPN readily invading and killing *R. robini*, reporting mortality up to 30%, with as many as 30 infective juveniles (IJ) in parasitized individuals. However, they reported an inverse correlation between EPN size and ability to infect the mites, which argues against the likelihood that the relatively large *S. feltiae* could directly attack and kill most soil mites. Similarly, although more Japygidae were detected in the untreated plots, the high motility of most diplurans should impede infection by EPN. Collembolan predators of nematodes are also well documented. Laboratory studies by Gilmore [65] revealed 10 of 12 tested Collembola species fed on nematodes, Thimm et al. [66] verified nematode consumption from springtail gut contents and nematophagy under natural conditions was ascertained [67]. However, there are no reports of nematode predation on Collembola to explain the apparent response to *S. feltiae* in this study.

Metabarcoding revealed more than 100 insect taxa, of which a considerable number are not soil-dwelling insects. This merits further investigation, because barcoding of soil DNA is probably a useful practice to detect insects that are not only residing in soil. A similar approach is applied to detect vertebrates such as amphibians by filtering water in multiple locations of a river [68,69]. As there is no comprehensive record of soil insects in Florida citrus orchards, this work is the first to assess a community that potentially supports the rich and abundant EPN guild on the peninsula [15]. The predicted broadly negative response of insect ASVs to the *S. feltiae* treatment was like that of microarthropods where fewer total insect reads could reflect an accumulation of non-significant reductions in many taxa. Given the high mobility of most insects, the reduction may represent emigration due to predator avoidance [70,71] in addition to predation. The insect with the strongest response was possibly misidentified as *Myrmica semiparasitica* (Hymenoptera: Formicidae), a small, social parasitic ant with a reported distribution from southeastern Canada and New England to Ohio and Illinois. Regardless, the results agree with past reports showing that EPN readily infect Formicidae in the laboratory [72] and reduce *Solenopsis* (Hymenoptera: Formicidae) populations when applied to field colonies [73,74]. However, we are not aware of reports that entomopathogenic nematodes infect ants under natural conditions. A failure of insects to respond to *H. bacteriophora* is consistent with its more rapid disappearance than *S. feltiae*. A significant increase in response to *H. bacteriophora* by the herbivorous, non-subterranean geometrid moth *Stamnodes affiliata* (Lepidoptera: Geometridae) may also have been a misidentification or perhaps the result of an indirect effect such as a reduction of a natural enemy.

Our data highlight both the potential and a serious shortcoming of metagenomic tools to study soil communities [75]. The broad MOTU array delimited by barcoding provided a more accurate representation of the species richness and identity in this orchard than could be reasonably done using other methods, even by expert taxonomists. If identified, the ecological function of responsive species can then be studied in detail. For example, we found evidence that many fungi and the bactivorous nematodes *Acrobeles complexus* and *Acrobeloides saeedi* were suppressed by EPN augmentation, which, if verified by further studies, may be found to involve the same or different mechanisms reported to affect plant parasitic nematodes. Further exploration at this site for *M. semiparasitica* or similar ants could resolve the identity of that species and whether it truly sustains EPN in Florida orchards. Nevertheless, we were unable to identify candidate natural enemies of EPN, partly because so few MOTU were identified at higher resolution than family. At least one mite (accession JX863034.1) in the Oppiidae may have preyed heavily on *H. bacteriophora*, while several other responsive but unidentified species in Table 3 may have functioned as predators or prey depending on the EPN species introduced into the soil. The absence of genomic records for most microarthropod species will obscure their identity in the foreseeable future because, while 55,000 species of mites were described by the turn of the last century [76], estimates of their species richness range from a few to many millions of species [77]. Most families in the NCBI database have critically low coverage, with the

vast majority of entries identified only at the family level. Despite this shortcoming, the metagenomic approach used here provided important insights into how soil communities respond to EPN augmentation. The difference in the rhizosphere competence of the two EPN appears to have been driven by superior predatory capacity of *S. feltiae* on mites and insects, which also extended the duration of non-target effects on other nematodes that are associated with elevated nematode abundance in soil [25,26,37]. Moreover, the minor (2.1%) contribution by significantly responsive species to treatment differences in the microarthropod abundance indicates that this major predator guild functions collectively and broadly, with few key species, to regulate a nematode equilibrium in soil.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12102502/s1, Figure S1: Redundancy Analysis (RDA), response of nematodes; Figure S2: Redundancy Analysis (RDA), response of microarthropods Table S1: NCBI, taxa detection; Table S2: log(natural) total nematodes versus Treatment, sampling Day, replication; Table S3: log(natural) native nematodes versus Treatment, sampling Day, replication.

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