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Posted Date: 14 October 2026

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Article

Metarhizium Anisopliae as a Microbial Control of Varroa Mites (*Varroa destructor*) in Honey Bee (*Apis mellifera*) Colonies

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Abstract

The potential of the fungal strain *Metarhizium anisopliae* ESF1 to control *Varroa destructor* was evaluated in a field trial conducted on honey bee colonies, with comparisons made to the miticide Apiguard. At the end of the 73-day experimental period, *M. anisopliae* demonstrated efficacy comparable to the chemical treatment in suppressing *V. destructor* populations. The number of mites per honey bee (*Apis mellifera*) was significantly reduced in colonies treated with the miticide Apiguard and the fungus *M. anisopliae*; however, mite infestations increased in the control colonies. Similarly, the number of mites in sealed brood was significantly higher in the control colonies than in those treated with the miticide and the fungus. Mites remaining in the colonies post-treatments (residual mites) were higher in the control colonies but reduced by 75.6% and 92.8% in colony treated with the fungus and miticide, respectively. Colony strength in terms of bee populations, brood production, and food reserves (pollen and honey) were similar between treatment groups. Overall, microbial control of *V. destructor* with *M. anisopliae* could be a promising alternative to the use of chemicals and a useful component of an Integrated Pest Management program for the beekeeping industry.

Keywords: *Metarhizium anisopliae*; *Varroa destructor*; fungus; honey bee; beekeeping

1. Introduction

The ectoparasitic mite *Varroa destructor* Anderson and Trueman (Mesostigmata: Varroidae) is widely regarded as the most significant threat to apiculture globally [1]. Colonies infested with parasitic mite will become weakened or collapse if left untreated [2]. Büchler et al. [3] reported that majority of colony losses caused by *V. destructor* occur in autumn or winter, primarily due to high infestation levels and the weakened health of adult bees. The unique biology of this parasite has complicated control efforts. Except for mated females, all developmental stages of the *V. destructor* reside in brood cells, where they feed on the fat body tissues and hemolymph of developing honey bees [*Apis mellifera* L. (Hymenoptera: Apidae)] [4,5].

Various strategies have been explored for *V. destructor* control, including biotechnical, genetic, biological, and chemical approaches [6,7]. Biotechnical methods (e.g., thermal treatments, cell size modifications, and mite trapping devices) are labor-intensive and yield varying levels of success [8,9]. Chemical treatments remain essential for colony survival, including breeding programs aiming to develop mite-tolerant bee stocks in North America and Europe [10,11]. Historically, miticides like tau-fluvalinate (Apistan®) and coumaphos (CheckMite+®) were widely used due to their cost-effectiveness, but a resistance in *V. destructor* populations has reduced their efficacy [12–14]. Moreover, these chemicals leave harmful residues in hive products such as wax and honey [15–17].

Other chemical agents, including flumethrin, cymiazole, and bromopropylate, also pose challenges due to residue concerns, making their registration difficult in North America [15,18]. These residues raise environmental and public safety concerns, impacting honey and wax quality, contaminating the environment, and exposing beekeepers to toxic substances. While thymol and amitraz are currently the most commonly used treatments, their application can negatively affect colony development, and the potential for resistance is a growing concern [17,19,20]. Organic acids like formic, and oxalic acids offer an alternative, but they require multiple applications for effective control, have temperature-dependent efficacy, and pose safety risks to beekeepers [21]. Essential oils, though promising, lack consistent efficacy [22]. Increasing miticide doses to counteract resistance is not feasible, as this would further harm honey bees and result in chemical accumulation in hives [23].

While significant interest still exists for the development of chemical controls of *V. destructor* biological control strategies are starting to be considered as the method with the highest potential for success [6]. Achieving sustainable *V. destructor* control through biological strategies would represent a significant advancement for maintaining healthy bee colonies. Multiple studies have investigated the use of entomopathogenic fungi, bacteria, and invertebrates to control *V. destructor* [24–26]. Previous studies have identified fungi such as *Hirsutella thompsonii* and *Metarhizium anisopliae* as potential biocontrol agents for *V. destructor* [24,27–29]. However, a practical and user-friendly method of applying these fungi in hives has yet to be developed. Given the increasing resistance to conventional miticides [20], developing alternative, environmentally friendly control strategies is essential for the sustainability of the beekeeping industry. *Metarhizium anisopliae* is a well-studied entomopathogenic fungus used for biological pest control. Its impact on honey bees (*Apis mellifera*) has been investigated in multiple studies [30–32], with most findings indicating minimal to no harmful effects under natural conditions [33].

The primary objective of this study was to evaluate the efficacy of *Metarhizium anisopliae* EFS1 in controlling *V. destructor* in honey bee colonies. EFS1 is a highly virulent strain capable of infecting and killing *V. destructor* through direct contact. Laboratory and field studies have shown significant mite mortality within a few days of exposure. EFS1 has shown minimal harmful effects on adult bees and brood when properly formulated and applied. Some strains of *Metarhizium*, including EFS1, show reasonable efficacy in the temperature ranges typical of beehives (~30–35°C), which is often a limitation for other entomopathogenic fungi. Efficacy was measured by (a) monitoring mite infestations on adult bees before and after treatments, (b) assessing mite populations in sealed brood cells, (c) evaluating residual mite populations in the hive after treatment as assessed with an amitraz-based contrast treatment, and (d) assessing colony growth and development. We compared the performance of *M. anisopliae* with the miticide Apiguard and an untreated control group.

2. Materials and Methods

2.1. Establishment of Honey Bee Colonies

Field trials were conducted during the summer of 2023 at the Florida A&M University (FAMU) Research and Cooperative Extension Center in Quincy, Florida. The study consisted of three treatment groups, each comprising 10 strong honey bee (*Apis mellifera*) colonies. To ensure uniformity in both bee population size and *V. destructor* infestation levels across treatments, we followed the procedures described by [34] and [24]. Briefly, colonies from the FAMU apiary, maintained in standard Langstroth hives, were de-queened, and adult bees were shaken into a large wooden cage (77.5 cm × 77.5 cm × 80.5 cm) constructed with wire-screened sides. The cage was placed in a refrigeration unit (4°C, 85% RH) for 24 hours to reduce the bees' activity and allow them to intermingle. Afterward, the bees were weighed and divided into packages of 0.92 kg. To minimize genetic variability, new queens reared from the same mother were introduced to each colony two weeks prior to treatment.

The experimental colonies were housed in new single-chamber Langstroth hive bodies, each containing two frames of brood, three frames of honey, one frame of pollen, two frames of foundation, and an internal feeder. To reduced drifting and better assess treatment effects, the 10

colonies in each group were spaced 200 meters apart, with each colony within a group separated by 3 meters [34,35].

2.2. Fungal Products and Spore Viability

The fungal spores of *Metarhizium anisopliae* ESF1 were sourced from Jaronski Mycological Consulting LLC (Blacksburg, Virginia, USA), with a concentration of 5.2×10^{10} spores/g and a viability rate of 94%. Additional cultures of the fungus were prepared in the Center for Biological Control laboratory at FAMU as needed. To culture *M. anisopliae*, the spores were suspended in 0.02% Silwet L-77® (Loveland Industries Inc., Greeley, CO) and spread onto 15 Petri plates (10.0 x 1.5 cm) containing Sabouraud maltose agar (SMAY), supplemented with 1% yeast extract (Difco, Detroit, MI). After 14 days of incubation at $27 \pm 1^\circ\text{C}$ and 85% relative humidity (RH), the conidia were harvested.

To assess conidia viability, spores were again suspended in 0.02% Silwet L-77® and spread onto three additional SMAY plates. These plates were incubated for 20 hours under the same temperature and humidity conditions. Post-incubation, lactophenol cotton blue stain (0.5% cotton blue) was applied to each plate to stain the conidia and halt further germination. The stained conidia were then evaluated using a phase-contrast microscope at 400x magnification. In each of the three droplets on the plates, the first 100 conidia observed were assessed for germination, totaling 300 conidia per plate. A conidium was considered viable if its germ tube was at least as long as the width of the conidium. Viability estimates for all fungal treatments were determined based on the proportion of conidia that had germinated after incubation, following the protocol by Goettel and Inglis [36].

2.3. Treatments for Field Trials.

Ten honey bee colonies were assigned to each of the three treatment groups. Treatment 1 (dust application) consisted of applying 10 g of *M. anisopliae* topically by separating the top bars and distributing the material directly over the brood cluster. A pre-measured 10 g of dry fungal spores was applied to each colony using an Exacticide applicator duster (DoMyOwn, Inc., Lawrenceville, GA). A second application was administered on day 21. Treatment 2 served as the positive control, where two trays of 25 g Apiguard® gel (thymol) were placed in each colony at the start of the treatment and again 14 days later. Treatment 3 was the untreated control group, which received no fungal or chemical treatments. The field trials lasted 73 days, with regular monitoring to assess mite populations and colony health. Colony performance metrics, including brood production and food reserves, were also evaluated throughout the trial.

2.4. Evaluation of the Efficacy of the Treatments and Mite Infestations of Adult Bees

To assess mite infestation levels in adult bees, we used the alcohol wash method as described by Delaplane et al. [37] and Kanga et al. [35]. Approximately 300 bees were brushed from the edge of the brood chamber in each of the 10 colonies per treatment into a 0.5-liter glass jar containing 70% ethanol. The jars were taken to the laboratory, where they were shaken for 2-3 minutes to dislodge the mites from the bees. The alcohol solution was then poured through a mesh screen into a white plastic tray to separate the bees from the mites. This process was repeated twice or until no more mites were present in the solution. Each bee was examined individually to ensure that all mites had been removed, and the mites were counted. The number of mites was then divided by the number of bees in the sample to calculate the mite infestation level (mites per adult bee).

2.5. Mite Infestations in Sealed Brood Cells

The number of mites within sealed brood cells was quantified at the onset of the field trials and reassessed at the conclusion of the 73-day experimental period. This was done by making two opposing diagonal transects across the capped cells of a randomly selected brood frame from each colony. A total of 200 sealed brood cells were opened along the transects on each frame. Both the

brood and any mites present were carefully removed and counted to assess the level of infestations within the sealed brood cells [38].

2.6. Residual Mite Population Levels in Bee Hives

To estimate the number of mites remaining in each colony at the end of the 73-day experimental period, all honey bee colonies were treated with Apivar strips (two per colony). A sticky trap was placed below the screen bottom board of each hive to capture post-intervention mite density. The number of mites caught on the sticky traps was recorded daily for 14 days after the experimental period. This method allowed for a reliable assessment of the remaining mite population after the experimental treatments were completed [38,39].

2.7. Evaluations of Colony Growth and Strength

The strength of each colony was assessed by visually inspecting both sides of each frame in the hive to estimate the area covered with adult bees and brood [40]. These estimates were recorded in tenths of frame equivalents for a standard 232 mm deep Langstroth frame [41,42]. Evaluations of colony strength were conducted 2 days prior to treatment application, again at the end of the 73-day experimental period, and once more before the treatment for residual mites.

2.8. Statistical Analysis

Mite infestations of adult bees (as determined through the alcohol wash method) and residual mites in the colonies post treatments were analyzed using repeated measures analysis [43]. Treatments were modeled as fixed effects, while date and date-by-treatment interactions were treated as random effects. To separate treatment means, a Tukey's studentized range test was employed. Mite infestations of adult bees and the percentage of sealed brood cells infested with *V. destructor* were arcsine transformed to meet the assumptions of normality prior to analysis. Additionally, mite infestations in sealed brood and colony strength were subjected to analysis of covariance, using pre-treatment data as the covariate [43], with treatment means compared with Tukey's studentized range test. Data on adult bee populations, brood, pollen, and honey were transformed to $\log(x + 1)$ as needed to fulfill the normality assumptions before analysis. For all statistical tests $\alpha = 0.05$ and means $\pm$ SE of the untransformed data were reported.

3. Results

3.1. Mite Infestation Levels in Honey Bee Colonies

The number of mites per adult bee were comparable across all treatment groups at the start of the experiment on day 1 (Table 1). However, significant differences in mite infestations per adult bee were observed between treatments at 73 days post-treatment ($F = 7.75$; $df = 2, 27$; $P = 0.0022$). The time effect, which measures within-treatment variability over time, was not significant ($F = 0.69$; $df = 5, 129$; $P = 0.6283$). In contrast, the time-by-treatment interactions were significant ($F = 1.89$; $df = 10, 129$; $P = 0.0525$). At the end of the 73-day experimental period, mite infestation levels were significantly reduced in the chemical treatment (Apiguard) ($t = 3.75$; $df = 27$; $P = 0.0009$) and the fungal treatment (*Metarhizium*) ($t = 2.90$; $df = 27$; $P = 0.0074$) compared to the control groups. Mite infestation levels were similar between Apiguard and *Metarhizium* treatments at the end of the experimental run ($t = -0.80$; $df = 27$; $P = 0.4296$). The patterns of mite infestations over time varied among treatments, with the control groups exhibiting the highest levels of mites on adult bees (Figure 1). These results indicated that both chemical and biological treatments can effectively reduce mite populations, highlighting their potential utility in managing *V. destructor* infestations in honey bee colonies.

Table 1. The number of mites per hundred adult bees (Mean ± SE) in honey bee colonies treated with the fungus *Metarhizium anisopliae* and the miticide Apiguard and untreated (control) before and at the end of the 73-day experimental period.

Number of mites per hundred adult bees (Mean±SE)			Residual mite levels (Mean±SE)	
Treatments	Before	After	Treatments	Mite drop
Control	0.81±0.12aA	1.18±0.11aB	Control	40.02±6.53a
<i>Metarhizium</i>	0.83±0.14aA	0.58±0.09bB	<i>Metarhizium</i>	9.78±2.72b
Apiguard	0.57±0.10aA	0.43±0.12bB	Apiguard	2.88±0.70b

Means within columns are not significantly different if followed by the same lowercase letter [$P > 0.05$; Tukey test (SAS Institute 2013). Mean within rows are not significantly different if followed by the same upper-case letter. Residual mites (average daily mite drop) over 14 days post treatments.

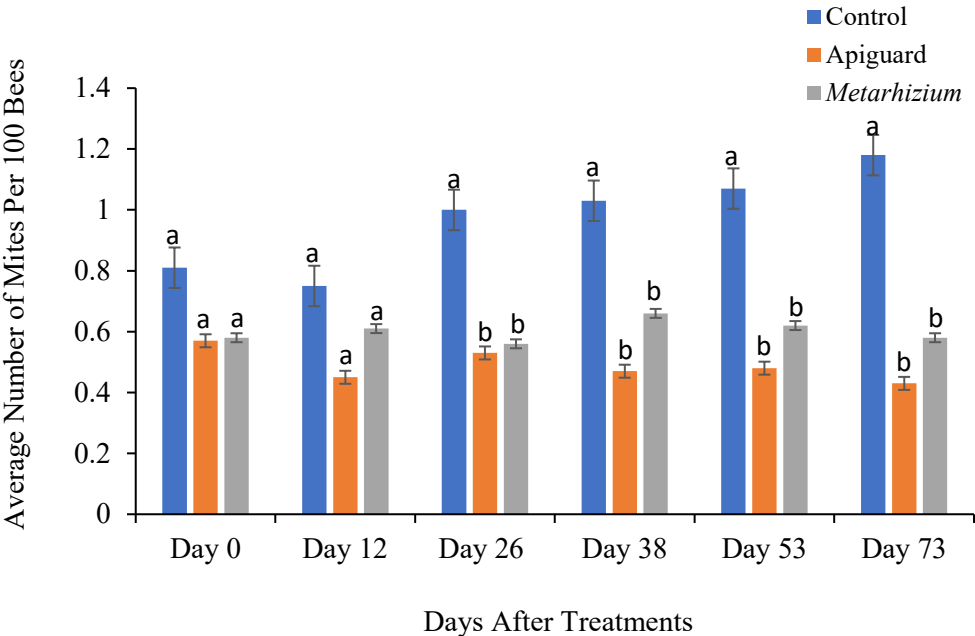


Figure 1. Mite infestations of adult bees (mean ± SE) in control, Apiguard and *Metarhizium* treated colonies during the 73-day experimental period. Different letters above bars within a day indicated treatment differences ($P < 0.05$, Tukey tests).

3.2. Post Treatment Mite Levels in Honey Bee Colonies

At the end of the 73-day experimental period, all colonies were treated with Apivar strips, and significant differences in the number of mites remaining in the colonies were observed between treatments ($F = 17.40$; $df = 2, 23$; $P < 0.0001$) over the 14 days of mite collections using sticky traps (Table 1). The time effect was also statistically significant ($F = 38.88$; $df = 13, 299$; $P < 0.0001$), as were the time-by-treatment interactions ($F = 2.92$; $df = 26, 299$; $P < 0.0001$). Control colonies had the highest number of mites remaining in the hives compared to those receiving fungal treatments (*Metarhizium*) ($t = 4.08$; $df = 23$; $P = 0.0005$) and chemical treatments (Apiguard) ($t = 5.63$; $df = 23$; $P < 0.0001$). Residual mite populations were similar between Apiguard and *Metarhizium*-treated colonies ($t = -1.48$; $df = 23$; $P = 0.1537$).

3.3. Mite Infestation Levels in Sealed Brood Cells

Mite infestations in sealed brood cells differed significantly among treatments across the experimental period ($F = 11.28$; $df = 2, 22$; $P = 0.0004$) (Table 2). Planned pairwise contrasts showed that infestations increased significantly over time in control colonies compared with both Apiguard ($t = 4.68$; $df = 22$; $P = 0.0001$) and *Metarhizium* ($t = 3.05$; $df = 22$; $P = 0.0059$) treatments. However, infestations did not differ significantly between the Apiguard and *Metarhizium* treatments ($t = -1.43$; $df = 22$; $P = 0.1665$). Thus, by the end of the 73-day period, both Apiguard and *Metarhizium* treatments significantly reduced sealed brood infestations relative to the untreated control.

Table 2. Number of mites in sealed brood of bee colonies treated with the miticide Apiguard, the fungus *Metarhizium anisopliae* and untreated (control) before and at the end of the 73-day Experimental Period.

Treatments	Number of mites per 100 sealed brood cells (Mean±SE)	
	Before	After
Control	1.48±0.45aA	2.03±0.005aA
Apiguard	0.70±0.24aA	0.11±0.08bB
<i>Metarhizium</i>	0.72±0.27aA	0.47±0.21bB

Means within columns are not significantly different if followed by the same lower-case letter. Means within rows are not significantly different if followed by the same upper-case letter [$P > 0.05$; Tukey test (SAS Institute 2013)].

3.4. Honey Bee Colony Growth and Development

The average number of bees in the colonies did not differ significantly between treatments at the end of the 73-day experimental period ($F = 2.99$; $df = 2, 13$; $P = 0.0856$). Adult bee populations showed a slight increase in the control group; however, these differences were not statistically significant when compared to the Apiguard treatments ($t = 1.80$; $df = 13$; $P = 0.0955$) and *Metarhizium* treatments ($t = 1.29$; $df = 13$; $P = 0.0796$). Populations of adult bees were comparable between the Apiguard and *Metarhizium* treated colonies ($t = 0.50$; $df = 13$; $P = 0.6255$) at the conclusion of the experiment. Overall, adult bee populations across all treatments did not exhibit significant changes compared to their initial population sizes at the start of the experiments (Figure 2-A).

Brood production in honey bee colonies did not differ significantly between treatments at the end of the experimental period ($F = 3.02$; $df = 2, 13$; $P = 0.0835$). Specifically, brood production in the control colonies was comparable to that in the Apiguard-treated colonies ($t = 0.95$; $df = 13$; $P = 0.3594$) and in the *Metarhizium*-treated colonies ($t = 1.47$; $df = 13$; $P = 0.1654$). Overall, brood development at the end of the 73-day experimental period was not significantly impacted by any of the treatments (Figure 2-B).

Pollen reserves in adult bees did not show significant differences between treatments at the end of the 73-day experimental period ($F = 0.09$; $df = 2, 13$; $P = 0.9110$). Specifically, the pollen levels in the control groups were comparable to those in the Apiguard-treated colonies ($t = -0.42$; $df = 13$; $P = 0.6815$) and the *Metarhizium*-treated colonies ($t = -0.08$; $df = 13$; $P = 0.9355$). Overall, all treatments experienced a decrease in pollen reserves compared to the start of the experiments (Figure 2-C).

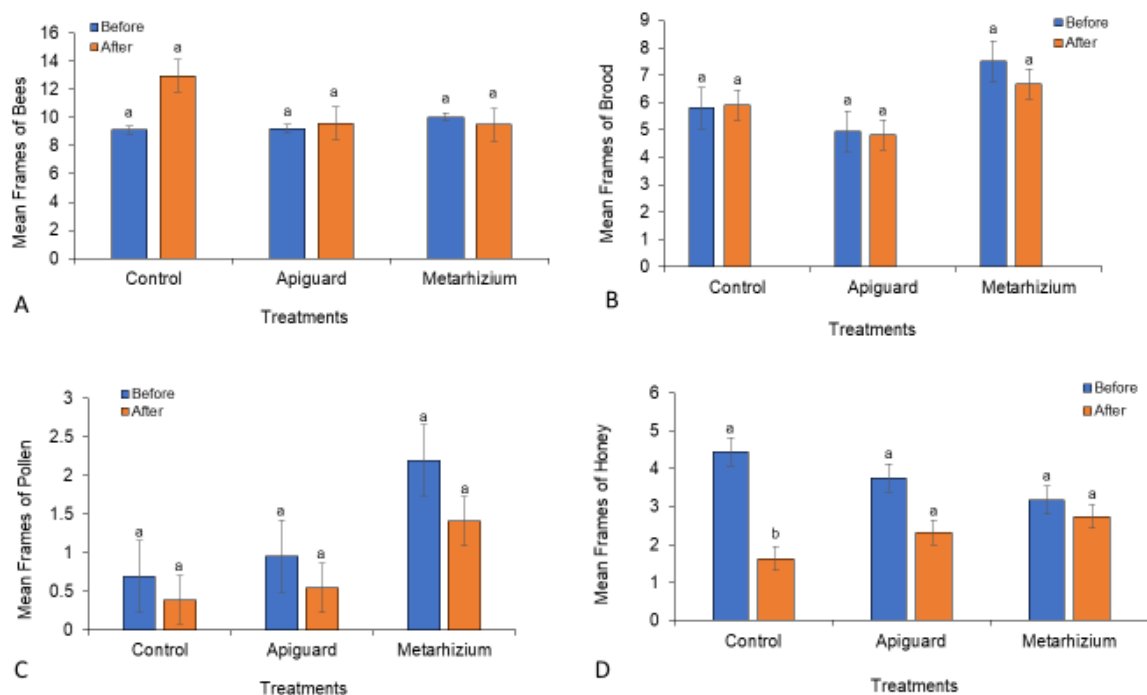


Figure 2. Adult bee populations (A), Brood production (B), Pollen reserves (C), and Honey reserves (D) in control, Apiguard, and *Metarhizium* treated colonies before and after treatments at the end of the 73-day experimental period. Different letters above bars within a day indicate treatment differences ($P < 0.05$, Tukey tests).

Honey reserves did not show significant differences between treatments at the end of the 73-day experimental period ($F = 0.94$; $df = 2, 13$; $P = 0.4168$). Specifically, honey reserves in the control groups were comparable to those in both the Apiguard-treated colonies ($t = -0.77$; $df = 13$; $P = 0.4553$) and the *Metarhizium*-treated colonies ($t = 0.67$; $df = 13$; $P = 0.5149$). Overall, while honey reserves decreased across all treatments compared to the start of the experiments, this decline was statistically significant only in the control colonies ($t = 2.60$; $df = 13$; $P = 0.0222$) (Figure 2-D).

4. Discussion

The fungal isolate *Metarhizium anisopliae* ESF1 effectively controlled *Varroa destructor* populations, demonstrating efficacy comparable to the chemical treatment Apiguard over the 73-day experimental period. These findings are noteworthy, as they expand the relatively small number of fungal isolates known to perform effectively against *V. destructor* under high-temperature conditions [24,35,44,45]. By the end of the study, when all colonies were treated with Amitraz, residual mite levels in fungal-treated colonies were similar to those observed in Apiguard-treated groups, further supporting the comparable effectiveness of these control strategies. Notably, only a limited number of *M. anisopliae* isolates have demonstrated such success in field conditions [27,35,38,45,46].

For fungal biocontrol agents to be effective, they must germinate and infect mites under brood nest conditions characterized by high temperatures (~35 °C) and low humidity [47]. Consistent with previous findings that only a few isolates tolerate such conditions [45], reductions in mite infestation in this study became evident approximately 26 days after treatment initiation. This lag aligns with earlier reports indicating that fungal pathogens typically require 2–3 weeks to significantly affect host populations under field conditions [48]. In contrast, untreated colonies exhibited continuous increases in mite populations, resulting in the highest infestation levels at the conclusion of the experiment.

While Apiguard effectively reduced mite infestation in adult bees and sealed brood cells, it was also associated with declines in colony strength, particularly in pollen and honey reserves. Although these reductions were not statistically significant, the overall trend suggests potential negative effects on foraging behavior. Previous studies have shown that thymol, the active component in Apiguard, can alter metabolic processes, including the downregulation of vitellogenin genes linked to foraging and longevity [49]. Additionally, pesticide exposure has been associated with adverse effects on honey bee health and development [17,50,51].

Mite infestations on adult bees declined significantly following application of *M. anisopliae*, while infestation levels in sealed brood cells were similar between fungal and Apiguard treatments and significantly lower than in controls. These results suggest that both treatments may interfere with mite reproduction. Supporting this, [52] reported behavioral avoidance of fungal spores by *V. destructor*, potentially influencing host selection. However, the mechanisms by which fungal or chemical treatments affect mites within brood cells or after emergence remain unclear.

Importantly, *M. anisopliae* ESF1 did not negatively impact colony strength, as measured by adult bee populations, brood production, or food reserves. This contrasts with some previous studies in which other *M. anisopliae* strains improved colony development [35,38]. In the present study, however, no significant differences in overall colony health parameters were observed, and the specific stage(s) of mite mortality—whether during dispersal, reproduction, or both—remain to be determined.

5. Conclusion

Current *Varroa destructor* management strategies rely heavily on chemical treatments, but the development of resistance presents an ongoing challenge for sustainable beekeeping [6]. The findings of this study demonstrate that *Metarhizium anisopliae* ESF1 is a promising biological control agent, offering efficacy comparable to conventional chemical treatments without negatively impacting colony health.

As interest in sustainable and integrated pest management (IPM) approaches grows, microbial control using entomopathogenic fungi represents a viable alternative. This approach is particularly valuable for organic, hobbyist, and commercial beekeepers seeking environmentally responsible solutions. Moreover, fungal pathogens may help delay or reduce the development of resistance in mite populations [53,54], with a low likelihood of resistance emergence [55].

Future research should focus on optimizing fungal formulations and application methods, as well as exploring complementary strategies such as secondary metabolites and molecular enhancements to improve efficacy [56–58]. Overall, *M. anisopliae* ESF1 and similar biocontrol agents hold significant potential to provide effective, sustainable, and environmentally sound solutions for managing *V. destructor* and supporting honey bee health.

Author Contributions: WD designed and performed the experiments, analyzed the data, and wrote the draft paper. L.H.B.K designed and performed the experiments, analyzed the data, and review, edit the paper. L.H.B.K was also responsible for obtaining funding. AG was involved in writing, advising, reviewing and editing the manuscript. Fungus for this project was provided by SJ and he also was involved in writing, reviewing and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the USDA-ARS-CMAVE FAIN. Project number 58-6036-2-010.

Institutional Review Board Statement: Not Applicable.

Informed Consent Statement: Not Applicable.

Acknowledgments: We are grateful to Rachel Mallinger, Marta Wayne, Blair Siegfried, Janice Peters, Jesusa Legaspi, Neil Miller, Nicholette Prince and Alejandro Bolques for providing technical support and useful discussions and reviews of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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