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Molecular Diagnostic Survey of Honey Bee, *Apis mellifera* L., Pathogens and Parasites from Arkansas, USA

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Original Article

MOLECULAR DIAGNOSTIC SURVEY OF HONEY BEE, *APIS MELLIFERA* L., PATHOGENS AND PARASITES FROM ARKANSAS, USA

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Abstract

Managed honey bee populations have fluctuated over the past several decades in the U.S. While a single factor has not been identified for these losses, the interaction between multiple biotic and abiotic stressors have been suggested to be responsible. Of major concern are several invasive parasite and pathogen species as well as colony management. A single honey bee colony often suffers from multiple harmful agents, that may act synergistically and cause greater declines in bee health. We conducted a survey to detect known and lesser-known honey bee parasites and pathogens. While previous research has primarily focused on commercially managed colonies, research is limited to pertaining parasite and pathogen prevalence in hobbyist managed colonies. Molecular diagnostics were used to screen 541 Arkansas (AR) honey bee colonies from 107 hobbyist beekeepers for eight A. *mellifera* parasite and pathogen species. Colony samples were obtained between 2015-2016 and represented forty-seven of the seventy-five AR counties. *Vairimorpha ceranae* (11.6% occurrence) and parasite *Varroa destructor* (49.4% occurrence) were relatively common in AR hobbyist colonies. Interestingly, the lesser-studied pathogenic trypanosome species, *Lotmaria passim*, was detected in 11.3% of the colonies and widespread in twenty of the forty-seven counties sampled. None of the honey bee pathogens *Vairimorpha apis*, *Spiroplasma apis*, *S. melliferum*, *Crithidia mellificae*, or the parasitic phorid fly, *Apocephalus borealis*, were detected in the colonies sampled. This study provides an extensive assessment of the parasite and pathogen species occurring at the AR state-level in hobbyist-managed honey bee colonies.

Keywords: *Apis mellifera*, Arkansas, molecular diagnostics, parasite, pathogen

INTRODUCTION

Total annual losses of honey bee, *Apis mellifera* L., colonies in the U.S. have averaged from 29% to 45% since 2008 (vanEngelsdorp et al., 2012; Fahey et al., 2019; Steinhauer et al., 2021). While a single factor has not been identified for these losses, research suggests that the interaction between multiple biotic and abiotic stressors contributes to elevated colony losses and reduced colony health (Potts et al., 2010; Core et al., 2012).

The U.S. migratory beekeeping industry, valued at several billion dollars annually, has expanded in recent decades, requiring more honey bee colonies to match agricultural demands (Calderone, 2012; Baylis et al., 2021). Honey bees are also important for honey production, valued at approximately $330 million (USD) annually (USDA-NASS, 2021).

Migratory beekeeping companies often transport poorly ventilated honey bee colonies thousands of kilometers during warm months, causing stress (Simone-Finstrom et al., 2016). Upon arrival, colonies from differing regions intermingle, potentially exchanging infectious agents (Klee et al., 2007; Simone-Finstrom et al., 2016). Hobbyists maintain smaller apiaries of fifty or fewer colonies and typically keep them stationary; this lack of exposure to colonies

from around the U.S. may limit introductions of newly emerging infectious agents in stationary colonies (Burgett et al., 1978). However, hobbyist beekeepers may utilize used equipment, packaged bees, and queens from other areas of the country, all capable of spreading infectious agents (Mutinelli, 2011). Beginning in 2009, the USDA Animal Plant and Health Inspection Service (APHIS) has conducted annual national surveys, most recently monitoring for several viruses, *Vairimorpha* (*Nosema*) (Nageli) (Microsporidia: Nosematidae) spp. (genus recently redefined by Tokarev et al., 2020), and two parasitic mite species. The surveys allow for extensive documentation of harmful infectious agents, but knowledge gaps remain, specifically for lesser-studied pathogen species (Fahey et al., 2019). Additionally, due to sampling limitations, individual states are not comprehensively surveyed, this is unfortunate as they could provide a more holistic understanding of honey bee harmful agents and their occurrence.

While studies have established that *Vairimorpha* spp. and *V. destructor* Anderson & Trueman are abundant and widelyspread in the U.S. (Core et al., 2012; Evans & Schwarz, 2011), numerous other species of honey bee pathogens and parasites lack adequate research (Chen et al., 2008; Evans & Schwarz, 2011). Among the lesser-studied agents are bacterial species *Spiroplasma apis* Mouches and *Spiroplasma melliferum* Clark, trypanosome species *Crithidia mellificae* Langridge & McGhee and *Lotmaria passim* Schwarz and parasitic phorid fly *Apocephalus borealis* Brues.

Pathogens *S. apis* and *S. melliferum* spread primarily during spring and summer, likely via fecal contamination from infected hosts on visited flower surfaces. These pathogens were isolated from the hemolymph and gut lumen of adult honey bees (Clark, 1977; Raju et al., 1981; Mouches et al., 1982), which displayed bloated abdomens filled with undigested pollen and body quivering (Mouches et al., 1982). Additionally, both pathogen species were detected in adjacent wild bees, which suggested a spillover effect of pathogens leading to infection in wild pollinator populations (Ravoet et al., 2014; Nanetti et al., 2021).

Trypanosomatid species *C. mellificae* and *L. passim* infect the rectum of honey bees, but the transmission mechanisms remain unknown and data on their occurrence in the U.S. is limited (Langridge & McGhee, 1967; Ravoet et al., 2015; Schwarz et al., 2015). However, the related species *Crithidia bombi* Lipa and Triggiani, a known harmful parasite of bumbles bees, has been shown to inhibit colony founding and reduce overall fitness in infected queens (Yourth et al., 2008). Studies indicate that *L. passim,* the predominant species in the U.S., occurs more frequently in managed populations than feral (Schwarz et al., 2015; Williams et al., 2019), and both species have shown an association with *V. ceranae* and are linked with increased winter mortality (Ravoet et al., 2013; Tritschler et al., 2017; Williams et al., 2021). Additionally, the detection of *C. mellificae* and *L. passim* other Hymenoptera populations suggests cross-infectivity (Ravoet et al., 2015; Tripodi et al., 2018).

Apocephalus borealis is a phorid fly known to parasitize bumble bees, paper wasps and recently honey bees (Core et al., 2012). There have been confirmed cases of *A. borealis* in honey bees in California (2009), South Dakota (2010), Oregon (2012), Washington (2013), Vermont (2013), Pennsylvania (2014), New York (2015), Virginia (2016), Maine (2016) and North Carolina (2016) (Core et al., 2012; ZomBee Watch, 2012; Sagili & Marshall, 2016). Nocturnal abandonment has been observed in parasitized honey bees, and the ultimate emersion of the fly emerges results in bee death (Core et al., 2012).

Taxonomic and morphological identification and detection of understudied internal parasites and pathogens can be tedious and unreliable due to small size and polymorphic variability (Schwarz et al., 2015; Szalanski et al., 2016). Using species-specific PCR primers and other molecular diagnostics, researchers achieve a high degree of success in detecting uncommon parasites and pathogens (Klee et al., 2007).

Limited research has focused on the occurrence

of parasites and pathogens in non-migratory honey bee colonies in the U.S., specifically at the State-level. Such studies are essential to understanding pathogen spread and evaluating the impact of migratory beekeeping on bee health. The objective of this study was to detect the presence and distribution of parasites and pathogens in Arkansas hobbyist-managed honey bee colonies.

MATERIALS AND METHODS

Sample collection

In 2015, the University of Arkansas Insect Genetics Lab (Fayetteville, AR) contacted via mail one-thousand AR hobbyist beekeepers using apiary registration information from the Arkansas State Plant Board (Little Rock, AR) to inquire about participation in a honey bee parasite and pathogen survey. Interested beekeepers were mailed collection kits, including 250 ml containers with 70% ethanol and protocol information instructing them to collect 30-50 adult worker honey bees from up to five colonies in their apiary. Samples were returned to the Insect Genetics Lab and stored at 22°C. In 2016, collection kits were mailed to all previously participating beekeepers to obtain a second round of samples.

Varroa mite detection

Varroa destructor mites were detected using a mite wash adapted from Oliver (2013). The mite wash utilized two 500 ml containers separated with a mesh-centered lid. Each colony sample was shaken in the mite wash to dislodge mites onto the opposite side of the mesh. Mites were counted and placed in 1.5 ml Eppendorf tubes with 70% ethanol for future research.

DNA extraction

Pooled DNA was extracted from 6-10 adult worker honey bees of each colony sample. Bees were placed on a paper towel for three hours at 22°C to evaporate ethanol preservative from the bees. DNA was extracted using a salting-out protocol with in-house reagents (Sambrook & Russell, 2001) per (Szalanski et al., 2016) and stored at -20°C.

Successful DNA extraction was confirmed via PCR amplification of a portion of the honey bee mitochondrial DNA (mtDNA) cytochrome oxidase I and II (COI-COII) region with the use of primers E2 and H2 (Tab. 1). PCR was conducted per Cleary et al. (2018) using the following thermocycler conditions: 5 min at 94°C; 40 cycles at 94°C for 45 seconds; 46°C for 1 min; 72°C for 1 min; and a final extension of 72°C for 5 min (Garnery et al., 1993). PCR amplicons for this and all subsequent molecular diagnostic assays were detected by subjecting PCR products to electrophoresis on a 2% agarose gel and visualized using a BioDoc-it™ Imaging System (UVP, Inc., Upland, CA). A 600-1200 bp amplicon indicated successful DNA extraction; the amplicon size variation is due to an intergenic spacer region which varies among honey bee lineages.

Molecular diagnostics

Vairimorpha (*Nosema*)

DNA samples were screened for *Vairimorpha* spp. using PCR primers NosemaSSU-1F and NosemaSSU-1R (Tab.1), a 222 bp amplicon for *V. apis* and a 237 bp amplicon for *V. ceranae* were amplified using the small subunit gene region specific for *Vairimorpha* mtDNA (Szalanski et al., 2014). Previously sequenced positive controls for both species were included in reactions and a negative control using PCR water. The thermocycler conditions were 2 min at 94°C, 40 cycles of 94°C for 45 seconds, 50°C for 1 min, 72°C for 1 min and a final extension of 72°C for 5 min (Szalanski et al., 2014).

Samples positive for *Vairimorpha* underwent a Restriction Fragment Length Polymorphism (RFLP) analysis per Szalanski et al. (2014) to distinguish the *Vairimorpha* species. The RFLP digestion utilized restriction enzymes *Dra I*, cutting only *V. ceranae* at 79 bp, and *Rsa I*, cutting *V. apis* at 130 bp. Samples were incubated overnight at 37°C, and products were detected with the use of the previously stated methods.

Table 1.
List of PCR primers used in molecular detection of parasites and pathogens in this study

Primer	Sequence	Reference
E2	F: 5'-GGCAGAATAAGTGCATTG-3'	A
H2	R: 5'-CAATATCATTGATGACC-3'	A
NosemaSSU-1F	F: 5'-ACAATATGTATTAGATCTGATATA-3'	B
NosemaSSU-1R	R: 5'-TAATGATATGCTTAAG TTCAAAG-3'	B
Phorid-rRNA-1F	F: 5'-GTACACCTATACATTGGGTTCGTACATT AC-3'	C
Phorid-rRNA-1R	R: 5'-GAGRGCCATAAAAGTAGCTACACC-3'	C
S.apis ITS-F	F: 5'-AATGCCAGAAGCACGTATCC-3'	D
S.apis ITS-R	R: 5'-GAACGAGATATACTCATAAGCTGTTACAC-3'	D
Ms-160 F	F: 5'- TTGCA AAAGCTGTTTTAGATGC-3'	D
Ms-160-R	R: 5'- TGACCAGAAATGTTTGCTGAA-3'	D
CBSSU rRNA F2	F: 5'-CTTTTGACGAACAACTGCCCTATC-3'	E
CBSSU rRNA B4	R: 5'- AACCGAACGCACTAAACCCC-3'	E
L.passim18S-F	F: 5'-AGGGATATTTAAACCC ATCGAAAATCT-3'	F
C.mel 474-F	F: 5'-TTTACGCA TGTCATGCATGCCA-3'	F

A: Garnery et al., 1993; **B**: Szalanski et al., 2014; **C**: Core et al., 2012; **D**: Schwarz et al., 2014; **E**: Schmid-Hempel & Tognazzo, 2010; **F**: Szalanski et al., 2016

Phorid fly

The PCR primers Phorid-rRNA-1F and Phorid-rRNA-1R were used to screen for Phorid rRNA, indicated by a 486 bp amplicon (Tab. 1). The following thermocycler conditions were used: 5 min at 94°C, 39 cycles of 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 1 min and a final extension of 72°C for 5 min (Core et al., 2012). A previously sequenced positive sample was included as a positive control.

Spiroplasma

Multiplex PCR using primers *S. apis* ITS-F, *S. apis* ITS-R, Ms-160 F, and Ms-160-R (Schwarz et al., 2014) were used to detect *S. apis* and *S. melliferum* (Tab. 1). Since the PCR primers used by (Schwarz et al., 2014) were developed for qPCR, we modified the thermocycler conditions to 2 min at 94°C, 39 cycles of 94°C for 45 seconds, 54°C for 1 min, 72°C for 1 min, and a final extension of 72°C for 5 min. Positive controls for *S. apis* (33834 ATCC) and *S. melliferum* (33219 ATCC) were acquired from a type strain from the American Type Culture Collection (ATCC, Manassas, VA). Samples positive for *S. apis* yielded a 190 bp amplicon, and samples positive for *S. melliferum* resulted in a 160 bp amplicon.

Trypanosomes

Multiplex PCR using primers CBSSU rRNA F2, CBSSU rRNA B4 (Schmid-Hempel & Tognazzo, 2010), and *L. passim* 18S-F was used to detect trypanosome pathogen species (Szalanski et al., 2016) (Tab. 1). The CBSSU rRNA primers amplified a small subunit gene yielding a 716-724 bp amplicon for trypanosome species, and the *L. passim* 18S-F primer amplified only

L. passim, indicated by a 499 bp amplicon. PCR was performed under the following conditions: 5 min at 95°C, 40 cycles of 30 seconds at 95°C, annealing for 30 seconds at 57°C and a final extension of 72°C for 5 min which was modified from the conditions used by Schmid-Hempel & Tognazzo (2010).

Samples positive for trypanosomes underwent a separate multiplex PCR using primers CBSSU rRNA P2 and CBSSU rRNA B4 and *C.mel* 474-F, which yielded a 245 bp amplicon for samples positive for *C. mellificae* under thermocycler conditions of 2 min at 94°C, 40 cycles of 94°C for 45 seconds, 55°C for 1 min and a final extension of 72°C for 5 min (Szalanski et al., 2016) (Tab. 1). Positive controls for *C. mellificae* (30254 ATCC) and *L. passim* (PRA-422 ATCC) were obtained from type strains.

Data analysis

Analyses utilized R version 4.0.5 (R Core Team, 2021) statistical software. The relative proportions of each detected parasite or pathogen (*V. ceranae*, *L. passim*, and *V. destructor*) were analyzed for independence between the years 2015 (n=435) and 2016 (n=106) with the use of Fisher's exact test (α=0.05) per McDonald (2014). A two-sample Z-test with continuity correction was used to compare the relative proportion of samples positive for *V. ceranae* in AR (n=541 11.65%) to previous state-level surveys in Virginia (n=293 69.28%) (Traver & Fell, 2011), New York (n=528 43.94%), and South Dakota (n=300 29.00%) (Szalanski et al., 2013).

RESULTS

From 2015 to 2016, 541 honey bee colony samples were received from 107 AR beekeepers (10.7% of surveyed beekeepers, 6.2% of registered beekeepers), representing forty-seven of the seventy-five AR counties. In 2015, 80.41% (n=435) of the samples were received, while 19.59% (n=106) were received in 2016. Of the eight species screened, *V. ceranae*, *L. passim*, and *V. destructor* were detected, while *V. apis*, *A. borealis*, *S. apis*, *S. melliferum* and *C. mellificae* were not detected.

Vairimorpha* (*Nosema*) *ceranae

Vairimorpha ceranae was detected in twenty-eight AR counties (Fig. 1) and 11.65% of the samples, with a significant difference in prevalence between 2015 (13.56%) and 2016 (3.77%) (Fisher's exact test, $p<0.05$). Compared to state-level surveys in Virginia (Proportion Z-test, $p<0.05$), New York (Proportion Z-test, $p<0.05$) and South Dakota (Proportion Z-test, $p<0.05$), the prevalence of *V. ceranae* was significantly different (Traver & Fell, 2011; Szalanski et al., 2013).

Lotmaria passim

Lotmaria passim was detected in 11.28% of the colony samples in twenty AR counties (Fig. 1). There was no significant difference in *L. passim* occurrence between 2015 (11.72%) and 2016 (9.43%) (Fisher's exact test, $p=0.61$).

Varroa destructor

V. destructor had the highest occurrence among the species tested, with 49.4% of the colony samples having one or more mite(s) present. *Varroa destructor* also had the widest distribution, occurring in forty-three of the forty-seven AR counties surveyed (Fig. 1). The prevalence of *V. destructor* did not significantly differ between 2015 (48.05%) and 2016 (54.72%) (Fisher's exact test, $p=0.23$). Within a single colony sample, *V. destructor* counts ranged between 0 to 86, with an average of 2.62 mites detected.

DISCUSSION

This study is among the first state-level surveys in the U.S. to examine several parasite and pathogen species in honey bee colonies. Furthermore, unlike previous studies that had targeted large-scale commercial apiaries and migratory colonies, this study focused on hobbyist-managed stationary colonies, providing new information pertaining to the spread and occurrence of parasites and pathogens. According to this survey, *V. ceranae*, *V. destructor*, and *L. passim* occur in AR hobbyist-managed honey bee colonies.

Compared to the national prevalence of

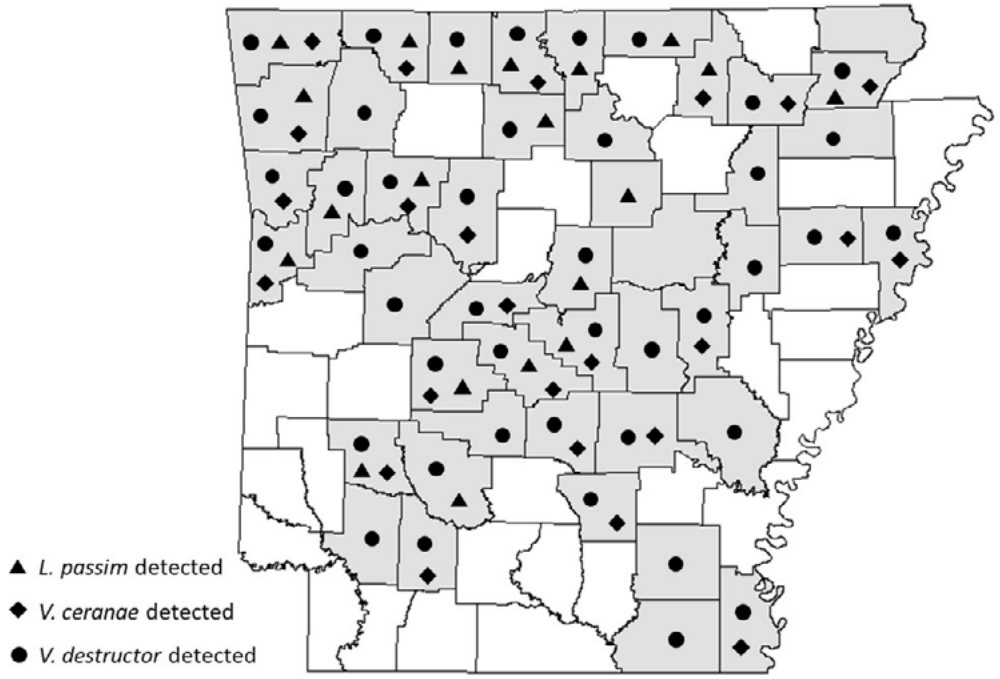

Fig. 1. Counties in Arkansas with colonies positive for *L. passim*, *V. ceranae*, and *V. destructor*. Counties in grey represent those from which honey bee colony samples were received.

Vairimorpha (n=947, 44.1%) and *V. destructor* (n=947, 89.5%), this survey had lower levels of *V. ceranae* (n=541, 11.6%) and *V. destructor* (n=541, 49.4%) (Fahey et al., 2019). The lower occurrence of *V. destructor* and *V. ceranae* in AR compared to national data may be explained by its inclusion of migratory honey bee colonies from different states. There is evidence that migratory honey bee colonies have higher instances of parasites and pathogens than stationary colonies (Alger et al., 2018). The national survey's data did not detect *V. ceranae* in the twenty-four AR samples received (Fahey et al., 2019). The limited number of colony samples from AR and other states in the national survey highlights the need for more comprehensive state-level honey bee health surveys.

Compared to other state-level surveys, the prevalence of *V. ceranae* was significantly lower in AR (11.6%) compared to surveys in Virginia (69.3%), New York (44%) and South Dakota (29%) (Traver & Fell, 2011; Szalanski et al., 2013). While the occurrence of *V. ceranae* within our survey is lower compared to that in other state-level surveys, it is important to note that exclusively hobbyist-managed colonies were sampled in our study. Research has indicated that *Vairimorpha* spp. is more prevalent in migratory colonies than in stationary colonies (Meixner & Conte, 2016). In addition, even though *Vairimorpha apis* had historically been the most common *Vairimorpha* species infecting U.S. honey bee colonies, it was not detected in the 541 honey bee colony samples, as studies suggest that *V. ceranae* is displacing *V. apis* (Chen et al., 2008). Lastly, our study found a significant difference in the occurrence of *V. ceranae* between 2015 and 2016.

The detection of *V. ceranae* and *V. destructor* across AR was unsurprising due to their wide distribution and documentation, but this study is among the first to report the occurrence of *L. passim* in AR. *Lotmaria passim* occurring in 11.3% of the colony samples indicates that the pathogen occurs outside migratory honey bee colonies in the U.S, and *L. passim* in twenty AR counties indicates that these are not isolated infections. Because *L. passim* is a more recently observed pathogen species, it is important to monitor its spread. This study further confirms the occurrence of *L. passim* in AR and supports

previous claims that *L. passim* is the predominant trypanosome species in honey bees, compared to *C. mellificae* (Schwarz et al., 2015; Williams et al., 2019). Future studies should focus on understanding the transmission of *L. passim* and *C. mellificae* and explore cross-infection to other Hymenopterans.

The prevalence of all three species, *V. ceranae*, *L. passim*, and *V. destructor*, was higher in 2016 compared to 2015, with a significant difference in *V. ceranae* prevalence. It is difficult to draw conclusions based on our dataset on differences in yearly prevalence due to uneven sampling and unknown colony sampling consistency between years. Future surveys should indicate whether subsequent samples are from newly established colonies, the origin of the colonies sampled and the success or failure of previously sampled colonies. This additional information will aid in a better understanding of the infection persistence among the parasites and pathogens tested between years.

None of the colony samples were positive for *S. apis* nor *S. melliferum*. The non-occurrence of *Spiroplasma* may be because it is a newly occurring pathogen in the U.S. Based on the survey data, it is likely that the AR hobbyist colonies have not yet been exposed to either species of *Spiroplasma*.

None of the colony samples tested positive for *A. borealis*. Because *A. borealis* causes hive abandonment, hive sampling is not the ideal sampling procedure (Core et al., 2012). Future sampling should target honey bees exhibiting such abnormal behavior as swarming porch lights at night. Furthermore, *A. borealis* has been cited primarily on the western and eastern coasts, while no neighboring states have identified the fly's presence, which suggests *A. borealis* has not expanded its distribution to AR to date (Core et al., 2012; ZomBee Watch, 2012; Sagili & Marshall, 2016).

As infectious agents continue to impact honey bee health negatively, monitoring efforts are necessary to determine parasite and pathogen occurrence and prevalence. Furthermore, monitoring lesser studied species is essential to assess their role in honey bee health. Previous studies have established that *V. destructor* and *Vairimorpha* spp. are abundant and widely occurring in commercial honey bee colonies (Chen et al., 2008; Evans & Schwarz, 2011), while this study indicates that *V. destructor, V. ceranae* and the lesser studied *L. passim* also occur in hobbyist-managed colonies.

This state-level multi-pest survey provides an extensive understanding of honey bee parasite and pathogen presence in AR. The results of this study may aid beekeepers in AR for future honey bee parasite and pathogen management decisions. In addition, results from this study will aid in understanding the spread of invasive parasites and pathogens in honey bees in the United States.

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