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FUNGICIDE CONTAMINATION REDUCES BENEFICIAL FUNGI IN BEE BREAD BASED ON AN AREA-WIDE FIELD STUDY IN HONEY BEE, *Apis mellifera*, COLONIES

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Fermentation by fungi converts stored pollen into bee bread that is fed to honey bee larvae, *Apis mellifera*, so the diversity of fungi in bee bread may be related to its food value. To explore the relationship between fungicide exposure and bee bread fungi, samples of bee bread collected from bee colonies pollinating orchards from 7 locations over 2 years were analyzed for fungicide residues and fungus composition. There were detectable levels of fungicides from regions that were sprayed before bloom. An organic orchard had the highest quantity and variety of fungicides, likely due to the presence of treated orchards within bees' flight range. *Aspergillus, Penicillium, Rhizopus*, and *Cladosporium* (beneficial fungi) were the primary fungal isolates found, regardless of habitat differences. There was some variation in fungal components amongst colonies, even within the same apiary. The variable components were *Absidia, Alternaria, Aureobasidium, Bipolaris, Fusarium, Geotrichum, Mucor, Nigrospora, Paecilomyces, Scopulariopsis*, and *Trichoderma*. The number of fungal isolates was reduced as an effect of fungicide contamination. *Aspergillus* abundance was particularly affected by increased fungicide levels, as indicated by Simpson's diversity index. Bee bread showing fungicide contamination originated from colonies, many of which showed chalkbrood symptoms.

Various beneficial fungi namely *Aspergillus, Penicillium, Cladosporium,* and *Rhizopus* (Gilliam et al., 1988) play a critical role in colonies of the honey bee, *Apis mellifera*. These fungi generate a natural defense shield for protection against potential microbial diseases, such as chalkbrood (*Ascosphaera apis*), and play a role in the manufacture of bee bread, which is a fungal fermentation product of pollen (Gilliam, 1979; Gilliam et al., 1989; Vásquez and Olofsson, 2009). Bee bread is a primary protein source necessary for the proper development and growth of adult bees and bee larvae. Fungicide spraying of orchard and agricultural crops appears to have the effect of reducing the density of naturally occurring spores (conidia) in the habitat, as well as contaminating pollen. As such, the amount of fungi that is carried into the bee colony is less and levels of beneficial fungi are suppressed (Yoder et al., 2012b). Fungicide residues are carried back into the colony via contaminated pollen and by the bees themselves (Kubik et al., 1999, 2000; Carlton and Jones, 2007; Alarcón

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et al., 2009; Škerl et al., 2009). A higher incidence of chalkbrood (*Ascosphaera apis*) has been reported in commercial bee colonies exposed to fungicide-sprayed areas (J. Finley, USDA-ARS, Tucson, AZ, and L. Crowder, Penasco, NM, personal communication, 2013).

In order to explore the impact of fungicides on bee bread mycoflora in the field, bee bread was sampled from colonies belonging to a commercial beekeeper who had rented colonies for pollination in almond orchards. Bee bread was sampled from the almond orchards, and later in the spring, when the colonies were moved into apples and some other fruits (all of the fruit orchards were treated with fungicide). The same group of colonies was sampled throughout the spring as they were transported to new orchards. The beekeeper was concerned that the fungicides might be exerting an impact on his colonies, and agreed to allow us to collect samples.

Not all bee colony fungi respond the same way to all fungicides (Yoder et al., 2012a); thus, the fungal composition in the bee bread mycoflora varies depending on which fungicides are sprayed in the habitats where bee colonies are located. This is highly relevant when moving honeybee colonies. When commercial beekeepers rent their colonies for pollination, they often move bees among different crops and different orchards to coincide with blooming times. Moving exposes the bee colonies to various different fungicides, with different modes of action, and chemical residue might accumulate in the beeswax and propolis (Mullin et al., 2010). Frequent fungicide exposure may affect the colony's fungal abundance and diversity, which may subsequently affect bee bread quality. The aim of this study was to clarify the relationship between fungicide exposure and fungal diversity in bee bread. The bee bread samples were cultured for fungi, and fungal diversity was correlated with the amount and types of fungicide residues present in the same samples. Those observations were combined with results from samples collected from bee colonies in Arizona, California, and Washington, including colonies not exposed to fungicides, in order to examine (1) regional differences in fungicide impact on bee bread mycoflora, and (2) extent that the composition of fungi in bee bread varies between different habitats and within the same apiary.

MATERIALS AND METHODS

Bee Bread Samples

Aseptic technique was followed throughout sample collection and processing, which involved using all autoclave-sterilized (121°C, 19 psi, 15 min) or manufacturer-sterilized (Fisher Scientific, Pittsburgh, PA) materials, powder-free gloves, and instruments. All solutions and preparation of media were conducted using glass equipment. Core samples of bee bread were collected by inserting a disposable, polyethylene 1-mL pipette tip (Fisher Scientific, Pittsburgh, PA), with a cut tip, into the center of bee bread honeycomb cells. One core sample was collected per cell ($n = 10$ per colony each from a different cell). The bee bread remained in the pipette tip, and each was placed into a separate 50-mL sterile polypropylene centrifuge tube (Fisher). Bee bread samples were collected from 10–20 colonies located in the almond orchards. The same colonies were followed as the bees were moved from almonds, to apples, to canola, over the spring months. The selection of cells for coring the bee bread was done using a randomized block design within each colony. The samples were placed into coolers containing Koolit cold packs (FDC Packaging, Medfield, MA) for transport, or shipment, to the lab. The samples did not make contact with the cold pack. Once in the lab, samples were stored at 4°C in a frost-free refrigerator (Fisher) for less than 24 h until processing.

All experimental work was conducted in a vertical laminar flow hood (Cole-Parmer, Vernon Hills, IL) that was wiped down daily with chlorine bleach (Clorox, Oakland, CA) and exposed to ultraviolet (UV) light. In the lab, pipette tips containing the core of bee bread were placed into a petri plate and the tip was sliced opened with a scalpel. The core sample was weighed with a microbalance (SD

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±0.2 μg precision and SD ±6 μg accuracy at 1 mg; Cahn Ventron Instruments, Cerritos, CA). All bee bread samples were standardized for mass to 0.5 mg. The core sample was then placed into a 1.5-mL microfuge tube (Fisher) with 1 mL deionized double-distilled (DI) water. The samples were mixed with a vortex (Scientific Industries, Bohemia, NY) to make a stock solution. Serial dilutions of the stock extract were made at 0.1, 0.01, and 0.001 in 1.5-mL microfuge tubes using DI water as the diluent. The transfer of solution from one tube to the next was carried out using a calibrated glass micropipette (accuracy ±0.25%, precision CV < 0.6%; Fisher), and the solutions were mixed using the vortex before transfer to the next tube (Brown, 2007). The samples were resuspended prior to plating by mixing with the vortex. The control was DI water without bee bread.

Fungal Analysis

Sets of agar plates were made with potato dextrose agar (PDA), modified potato dextrose agar (PDA acidified to pH 5.5 with lactic acid, MPDA), and modified Melin–Norkrans agar (MMN; Difco, Fisher) poured in 100 × 15-mm petri plates and then used after the agar solidified. Incubation conditions were 30 ± 1°C (regular bee colony temperature that is maintained by bee activity; Cooper, 1980; Chiesa et al., 1989) and darkness in a programmable incubator (< ± 0.5°C; Fisher). One-milliliter bee bread solutions were spread over the agar surface, at one dilution per petri plate. Plates were examined daily for developing hyphae. Fungal colonies were counted with a colony counter (Bantex Co., Burlingame, CA). Each fungal colony was referred to as a new isolate and was purified by subculturing hyphal tips. Identification of the fungus was performed at 1000× under oil once culture and spore (i.e., conidia, phialide) characteristics appeared (Barnett and Hunter, 1998). Identification was also based by comparison to pure cultures. Images of the culture plates were taken with a SPOT digital camera (Diagnostic Systems Laboratories, Webster, TX) attached to an Olympus stereoscope (Olympus America, Center Valley, PA). Figures 1 and 2 were constructed using Adobe Photoshop CS (Adobe Systems, San Jose, CA).

Sample Sizes and Statistics

Each set of dilutions was conducted in triplicate, using a different 0.5-mg sample of bee bread, from the same bee colony, per replicate. There were several bee colonies within each apiary. Bee colonies were selected at random. Three bee bread samples were taken from within each bee colony, each from a different frame. Data were compared using an analysis of covariance (ANCOVA; $p = .05$; SPSS 14.0, Microsoft Excel and Minitab, Chicago, IL) using an arcsine transformation in the case of percentage (Sokal and Rohlf, 1995). Simpson's diversity index (Simpson, 1949) was used to measure genera diversity and was calculated against the total number of fungal isolates (i.e., colonies). A low Simpson's diversity index implies that there is a high probability that when selecting two fungi at random from within the same bee colony that these will be the same genera. Conversely, a high Simpson's diversity index implies that when selecting two fungi from the same bee colony there is a high probability that these will be different genera. For the Simpson's index, all members of the same genus were treated as one group—that is, all *Aspergillus* species were combined.

Fungicide Residue Detection and Disease Diagnosis

A corresponding analysis of these bee bread, as well as pollen (subsequently bee bread), samples was carried out at the U.S. Department of Agriculture (USDA) National Science Laboratory (Gastonia, NC) according to Wu et al. (2011). Briefly, pesticide residues were analyzed using QuEChERS extraction and gas chromatography/mass spectroscopy (GC/MS) detection (Agilent 1100 LC, 6890 GC, 5975 mass-selective detector) set in in electron impact (EI) or negative chemical ionization

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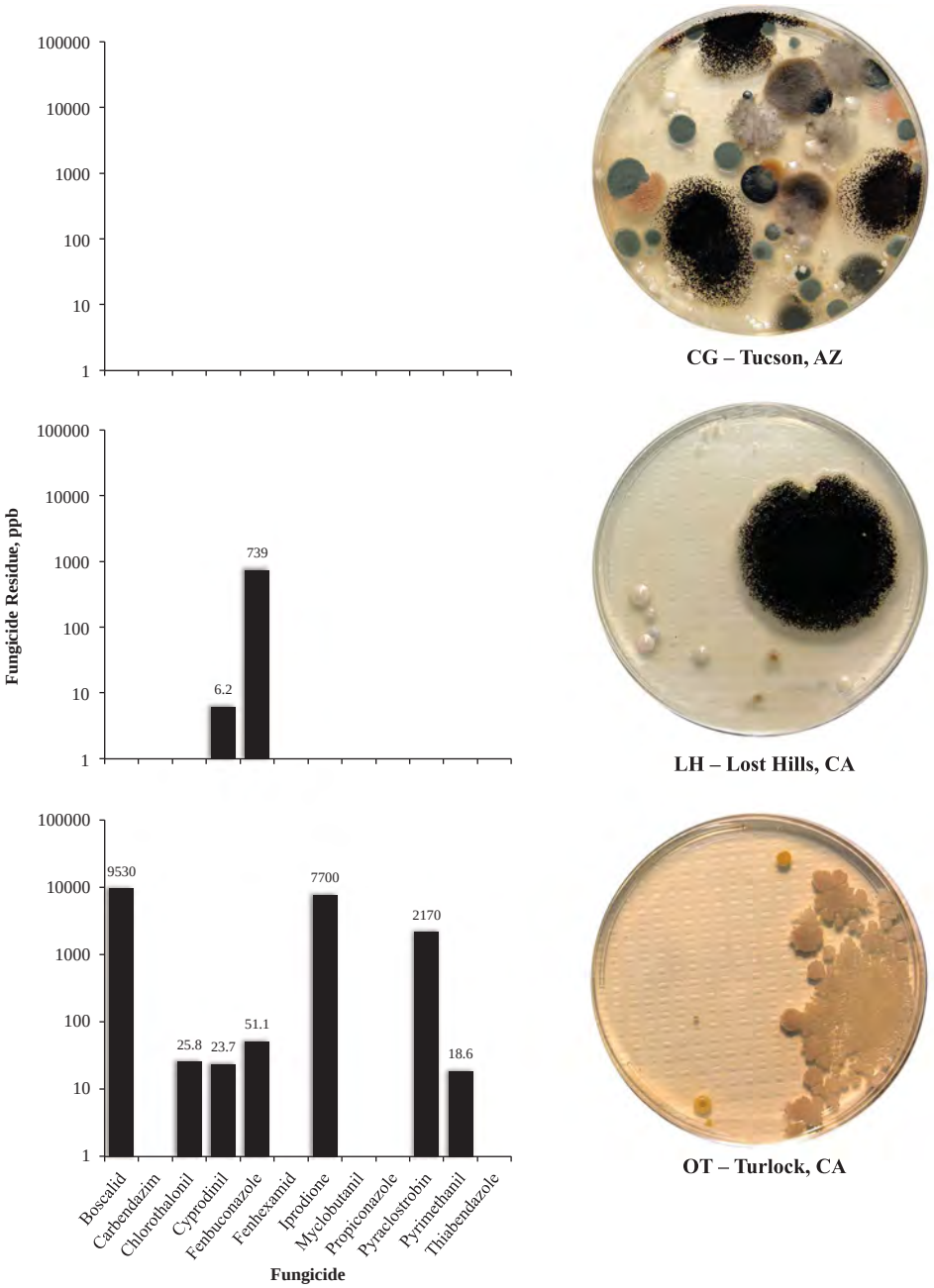

FIGURE 1. Fungicide residue analysis (± SE < 3.1) and fungal cultures of bee bread for select colonies from Arizona and California. CG, no history of fungicide spraying (no spray); LH, nontarget orchard, fungicides sprayed before bloom but surrounded by sprayed orchards (indirect spray); OT, an orchard that is sprayed directly with fungicide (direct spray). Plates were chosen randomly (incubation: potato dextrose agar, 30°C, darkness, 48 h, plated undiluted, 0.5 mg of bee bread/1 mL water). Photo credit: K. Gribbins, Department of Biology, Wittenberg University, Springfield, OH (color figure available online).

(NCI) mode. Quantification and identification of pesticide residues were based on a matrix match and ion ratio of calibrated authentic standards. Samples were run in triplicate. In some instances where chalkbrood and other disease symptoms were noted, samples were sent to the USDA Bee Research Laboratory (Beltsville, MD). A standard bee disease diagnosis based on microscopic examination for bee pathogens encompassing bacteria, viruses,

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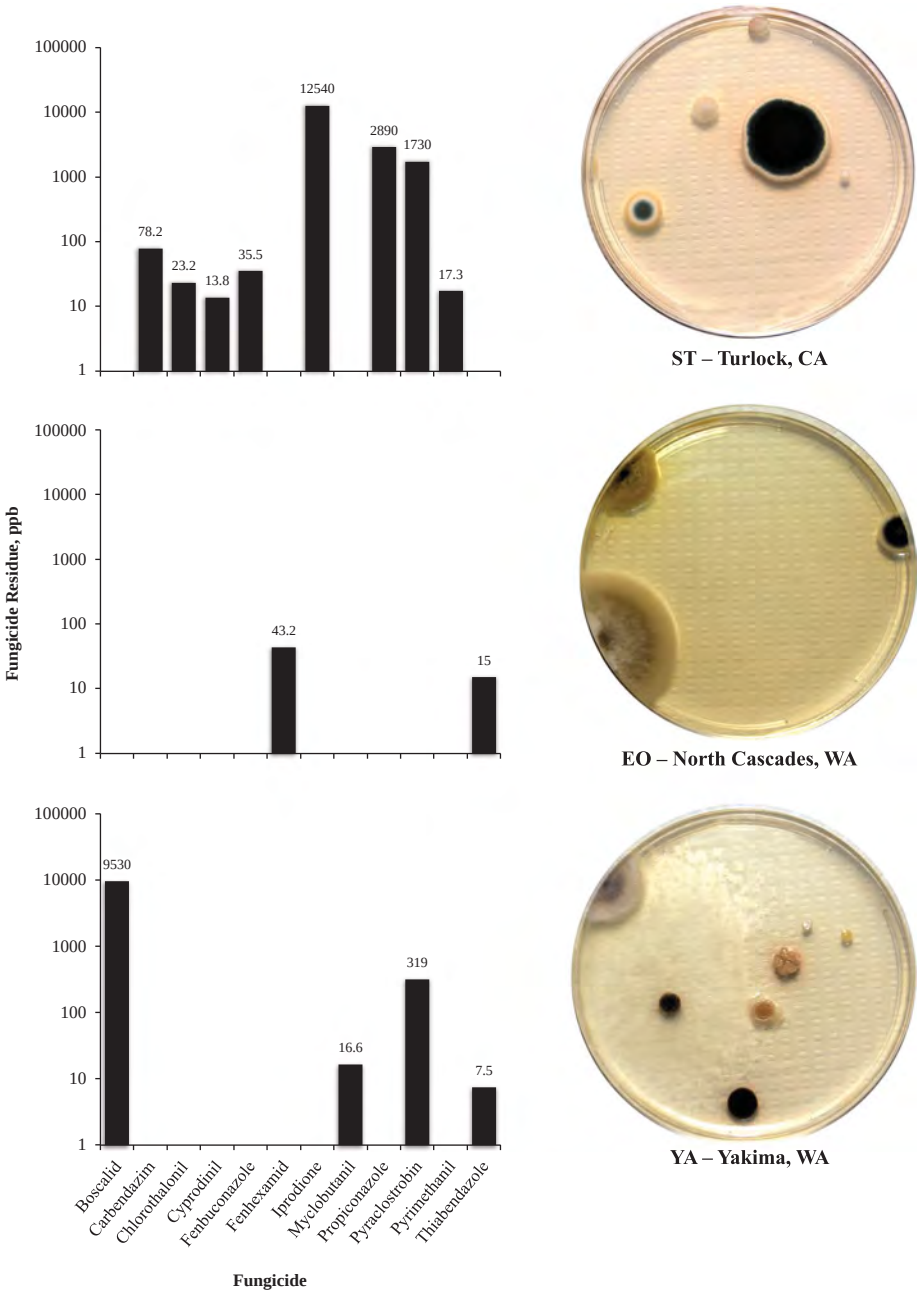

FIGURE 2. Bee bread samples analyzed for fungicide residues (± SE < 2.4) and fungus culturing for select colonies from California and Washington. ST, organic orchard surrounded by sprayed orchards (indirect spray); EO, national forest, but bee colonies had been previously exposed to sprayed orchards; YA, orchard that had direct fungicide spraying. Incubation: potato dextrose agar, 30°C, darkness, 48 h, plated 0.5 mg of bee bread/1 mL water (undiluted). The choice of plates was random, selecting for neither best nor worst examples of fungi in the batch. Photo credit: K. Gribbins, Department of Biology, Wittenberg University, Springfield, OH (color figure available online).

fungi, and protozoans was conducted on those samples and was also based on observation of these bee colonies for disease symptoms in the field (Shimanuki and Knox, 2000).

RESULTS

Colony history and notes pertaining to sites of bee bread collection are in Table 1.

TABLE 1. Background Information on the Honey Bee (*Apis mellifera*) Colony Sampled Over Several Months for Bee Bread

State	Apiary ID	Sample date	Main pollen source	Additional comments
Arizona	CG-Tucson	19 Apr 10	Wildflower	Little or no fungicide exposure Active colonies Not an intensive agricultural area No colonies positive for chalkbrood
California	LH-Lost Hills	4 Mar 10	Almond	Indirect fungicide exposure: surrounding areas treated Active colonies Likely subjected to many types of pesticides Some colonies positive for chalkbrood
	OT-Turlock	24 Feb 10	Almond	Direct fungicide and pesticide exposure Active colonies Some colonies positive for chalkbrood
	ST-Turlock	9 Mar 10	Almond	Indirect fungicide and pesticide exposure: surrounding areas treated Active colonies Certified organic orchard Some colonies positive for chalkbrood
	TB-Terra Bella	27 Jan 10	Almond	Direct fungicide and pesticide exposure Colonies died from unknown causes
Washington	EO- Yakima	27 Jan 10	Home apiary some orchards nearby, mostly spring fruit	Direct fungicide and pesticide exposure Some colonies died from unknown causes
	YA- Yakima	7 Apr 10	Apple	Direct fungicide and pesticide exposure Active colonies Some colonies positive for chalkbrood

Note. Active colonies were those that showed abundant pollen in pollen traps as an indicator of intense foraging.

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Fungicide Residue Analysis and Exploratory Observations of Fungal Cultures

No fungicide residues were detected in the bee bread from Arizona (CG) that had no history of fungicide spraying (Figure 1 and Table 1). In contrast, bee bread samples from Turlock, CA (OT and ST), displayed significantly the highest and most diverse fungicide levels among the different sites (Figures 1 and 2). The residue analysis was consistent between the two Turlock sampling sites (Table 1), except carbendazim and propiconazole were detected in the organic orchard (Figure 2). At the Turlock site directly sprayed with fungicide (OT), the levels of boscalid, cyprodinil, and fenbuconazole were significantly higher than at the organic orchard (ST) site in Turlock. Other residues (chlorothalonil, pyraclostrobin, pyrimethanil) were similar, and one, iprodione, was actually significantly higher in the organic orchard site (Figures 1 and 3). A high level of fenbuconazole, but lower level of cyprodinil, and absence of all the other fungicide residues are the distinguishing features of the Lost Hills, CA, samples, which were significantly lower compared to samples from Turlock area (Figure 2). Samples from Washington showed significantly lower amounts of fungicide residues than the Turlock, CA, sites (Figures 1 and 2). Both Turlock sites and the Yakima site consistently showed boscalid and pyraclostrobin; the myclobutanil and thiabendazole were distinctive fungicide residues for the Yakima site (Figures 1 and 2). Samples coming from the North Cascades, National Forest, in Washington also showed the presence of thiabendazole, as in Yakima (except significantly twice higher the amount), and a distinct one, fenhexamid (Figure 2).

The observable difference among field sites (Figures 1 and 2) in the number of fungal colonies on the plates is strictly quantitative, because the same 0.5-mg mass of bee bread was plated in each individual experiment. Figures 1 and 2 show petri plates of fungal

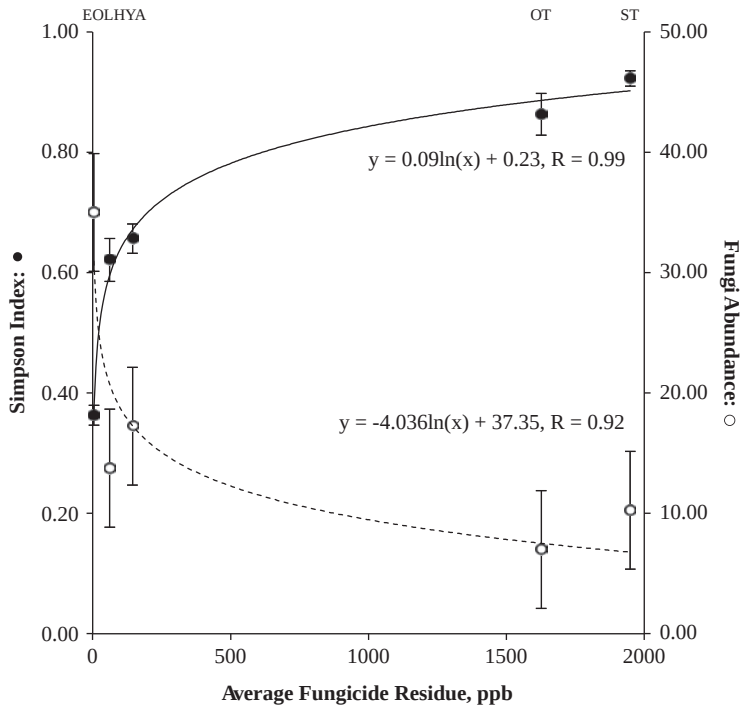

FIGURE 3. Relationship between the mean Simpson's diversity index and the mean number of fungal isolates (fungal abundance) as a function of the mean quantity of total fungicide. The mean Simpson's diversity index is the mean of the diversity index across all colonies at a particular site, and likewise for the mean number of fungal isolates (taken from Tables 2–5). Mean fungicide quantity is the mean of all fungicide residues in ppb at a particular site (taken from Figures 1 and 2). Colony designation is given across the top of the figure: EO, North Cascades; LH, Lost Hills; YA, Yakima; OT, Turlock; ST, Turlock. Baseline values for CG, Tucson: the mean Simpson's diversity index = 0.75 ± 0.01 (SE), the mean number of fungal isolates = 24.38 ± 2.13 (SE), and the mean quantity of fungicide was not detected. Colony histories are per Table 1.

cultures on potato dextrose agar media. Nearly identical images of fungal cultures similar to that found on potato dextrose agar were observed when the bee bread was cultured on modified potato dextrose agar, pH 5.5. This was also the case when bee bread was cultured on modified Melin–Norkrans agar (data not presented), thus indicating that these fungi are probably not fastidious (i.e., have no specific nutrient requirements) and are readily culturable. The culture plate of the one chosen from the batch representing the direct sprayed orchard in Turlock, CA (OT), was highly reminiscent of how the majority of those plates appeared by displaying mostly bacterial colonies, and few, if any, fungi (bottom, Figure 1). Therefore, fewer fungal colonies are visible on fungal culture plates from bee bread that contains detectable levels of fungicide residues than from bee bread where no fungicide residues are detected. This is even true for locations where the fungicides were not sprayed directly. When combined with bee colony history, fungicide residue, and fungal culture analysis of samples from Turlock, this is what was found in the California organic orchard (ST; Figure 2) plates. Most of the bee colonies that had detectable levels of fungicide residues demonstrated evidence of chalkbrood symptoms (Table 1; Figures 1 and 2), except for the Arizona colonies, where no chalkbrood was seen.

Mycoflora of Bee Bread Fungi From Arizona Honey Bee Colonies

Analysis of bee bread samples showed *Aspergillus*, *A. niger*, *Penicillium*, and secondarily *Rhizopus* were consistently the major isolates (Table 2). *Cladosporium*, *Mucor,* and *M. sterilia* (Table 2) were similar to amounts of *Rhizopus* (Table 2), and these were present in most, but not all, of the bee bread samples.

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TABLE 2. Mycoflora of Arizona Bee Bread Samples From Select Colonies

	Number of isolates (%) from bee bread from Arizona colonies:								
Fungi	CG5	CG7	CG9	CG13	CG16	CG17	CG18	CG20	Controls
Absidia	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Alternaria	0 (0)	1 (5)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)
Aspergillus	4 (19)	3 (15)	5 (22)	5 (19)	8 (26)	3 (16)	7 (25)	6 (22)	0 (0)
Aspergillus flavus	0 (0)	1 (5)	0 (0)	1 (4)	0 (0)	1 (5)	2 (7)	0 (0)	0 (0)
Aspergillus niger	5 (24)	4 (20)	6 (26)	3 (12)	6 (19)	3 (16)	5 (18)	5 (19)	0 (0)
Aureobasidium	0 (0)	0 (0)	0 (0)	1 (4)	2 (7)	0 (0)	0 (0)	1 (4)	0 (0)
Bipolaris	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)
Cladosporium	0 (0)	0 (0)	1 (4)	2 (8)	4 (13)	1 (5)	2 (7)	3 (11)	0 (0)
Fusarium	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)
Mucor	1 (5)	0 (0)	2 (9)	0 (0)	0 (0)	1 (5)	2 (7)	2 (7)	0 (0)
Mycelia sterilia	2 (10)	0 (0)	0 (0)	2 (8)	1 (3)	1 (5)	1 (4)	1 (4)	0 (0)
Paecilomyces	0 (0)	1 (5)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Penicillium	5 (24)	6 (30)	8 (35)	7 (27)	7 (23)	6 (32)	4 (14)	7 (26)	0 (0)
Rhizopus	3 (14)	4 (20)	1 (4)	2 (8)	1 (3)	2 (11)	3 (11)	1 (4)	0 (0)
Scopulariopsis	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Trichoderma	1 (5)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)	1 (4)	1 (4)	2 (100)
Total isolates	21	20	23	26	31	19	28	27	2
Simpson index	0.76	0.74	0.67	0.82	0.74	0.78	0.73	0.77	
SE	0.014	0.011	0.013	0.010	0.011	0.014	0.015	0.011	

Note. Colony designations are per Table 1. Number of isolates is combined from potato dextrose agar, modified potato dextrose agar (pH 5.5), and modified Melin–Norkrans agar, dilution factor = 10. Controls are agar plates streaked with water containing no bee bread.

There were a few isolates of *Absidia*, *Alternaria*, *A. flavus*, *Aureobasidium*, *Bipolaris*, *Fusarium*, *Paecilomyces*, *Scopulariopsis,* and *Trichoderma* that were recovered as minor components and their occurrence was mixed. The percentage composition of the individual fungal components of the bee bread varied in that no two bee colonies were identical (Table 2), except for the presence of *Aspergillus* and *Penicillium* predominating . These differences were reflected by differences in Simpson's diversity index (Table 2). Bee bread that had the largest number of isolates (CG16) did not display greater diversity of fungal components compared to bee bread that contained the significantly lower number of isolates (CG17); both contained nine different fungal taxa.

Mycoflora of Bee Bread Fungi from California Honey Bee Colonies

Total number of fungal isolates from all bee bread samples from California bee colonies (Tables 3 and 4) was significantly less than the total number of isolates that were recovered from samples from the Arizona bee colonies (Table 2). Of the data presented in Tables 3 and 4, no two bee colonies were completely alike with regard to percent composition of individual fungal components . These between-colony differences were evident by differences in Simpson's diversity index (Tables 3 and 4). In the Lost Hills samples, *Aspergillus*, *A. niger*, and *Penicillium* were the consistent, major isolates, while *Rhizopus* and *Cladosporium* also appeared with regular frequency. The *A. flavus*, *Geotrichum*, *Fusarium*, *M. sterilia*, *Paecilomyces*, and *Trichoderma* were variable components (Table 3). *Aspergillus* and *Penicillium* were the consistent major isolates from the OT bee colonies; the amounts of *A. niger* were significantly less than the amounts of *A. niger* in the samples from Lost Hills. *Cladosporium* and *Fusarium* were secondary, irregular components, and *Alternaria*, *A. flavus*, *Aureobasidium*, *Mucor*, *M. sterilia*, and *Trichoderma* were variable components (Table 3). In addition, from Turlock, *Aspergillus*, *Cladosporium*, and *Penicillium* were the major, consistent isolates found in the bee bread from the organic orchard (Table 4). The percent of *A. niger* was significantly lower than the amounts of *A. niger* from the other Turlock site (Table 3). The *A. flavus*,

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TABLE 3. Mycoflora of Select California Bee Bread Samples

	Number of isolates (%) from bee bread from California colonies:								
Fungi	LH5	LH11	LH 19	LH20	OT5	OT6	OT18	OT20	Controls
Alternaria	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (14)	0 (0)	0 (0)	0 (0)
Aspergillus	4 (31)	3 (19)	2 (17)	3 (21)	1 (20)	1 (14)	2 (20)	1 (16)	0 (0)
Aspergillus flavus	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16)	0 (0)
Aspergillus niger	5 (39)	6 (46)	4 (33)	6 (43)	1 (20)	0 (0)	1 (10)	1 (16)	0 (0)
Aureobasidium	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)
Cladosporium	1 (8)	0 (0)	2 (17)	1 (7)	1 (20)	1 (14)	0 (0)	0 (0)	0 (0)
Fusarium	0 (0)	1 (6)	1 (8)	0 (0)	1 (20)	1 (14)	0 (0)	0 (0)	0 (0)
Geotrichum	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Mucor	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)
Mycelia sterilia	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (16)	0 (0)
Paecilomyces	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Penicillium	2 (15)	2 (13)	1 (8)	2 (14)	1 (20)	1 (14)	4 (40)	1 (16)	0 (0)
Rhizopus	1 (8)	2 (13)	0 (0)	1 (7)	0 (0)	2 (29)	0 (0)	1 (16)	0 (0)
Trichoderma	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	0 (0)
Total isolates	13	16	12	14	5	7	10	6	0
Simpson index	0.53	0.61	0.76	0.37	0.90	0.95	0.80	0.80	
SE	0.041	0.032	0.033	0.037	0.039	0.018	0.025	0.056	

Note. Colony histories are in Table 1. Number of isolates is from potato dextrose agar, modified potato dextrose agar (pH 5.5), and modified Melin–Norkrans agar combined, dilution factor = 10. Controls, agar plates streaked with plain water without bee bread.

TABLE 4. Mycoflora of Select California Bee Bread Samples

	Number of isolates (%) from bee bread from California colonies:								
Fungi	ST4	ST6	ST12	ST23	TB1	TB2	TB3	TB4	Controls
Aspergillus	1 (13)	2 (17)	1 (11)	2 (17)	3 (19)	2 (18)	2 (20)	4 (31)	0 (0)
Aspergillus flavus	0 (13)	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Aspergillus niger	1 (13)	1 (8)	0 (0)	0 (0)	3 (19)	2 (18)	3 (30)	4 (31)	0 (0)
Aureobasidium	1 (13)	0 (0)	0 (0)	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)
Bipolaris	1 (0)	0 (0)	2 (22)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Cladosporium	2 (25)	1 (8)	1 (11)	3 (25)	1 (6)	1 (9)	0 (0)	1 (8)	0 (0)
Fusarium	1 (13)	2 (17)	0 (0)	2 (17)	0 (0)	1 (9)	0 (0)	0 (0)	0 (0)
Mucor	0 (0)	1 (8)	0 (0)	1 (8)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)
Mycelia sterilia	0 (0)	0 (0)	1 (11)	1 (8)	0 (0)	1 (9)	0 (0)	0 (0)	0 (0)
Paecilomyces	0 (0)	1 (8)	0 (0)	1 (8)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)
Penicillium	1 (13)	2 (17)	2 (22)	1 (8)	3 (19)	2 (18)	3 (30)	3 (23)	0 (0)
Rhizopus	0 (0)	1 (8)	0 (0)	0 (0)	2 (13)	2 (18)	2 (20)	1 (8)	0 (0)
Scopulariopsis	0 (0)	1 (8)	1 (11)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Trichoderma	0 (0)	0 (0)	1 (11)	0 (0)	1 (6)	0 (0)	0 (0)	0 (0)	0 (0)
Total isolates	8	12	9	12	16	11	10	13	0
Simpson index	0.93	0.92	0.94	0.89	0.84	0.85	0.69	0.60	
SE	0.016	0.011	0.012	0.012	0.017	0.020	0.025	0.034	

Note. The colony histories are in Table 1. Number of isolates is from potato dextrose agar, modified potato dextrose agar (pH 5.5), and modified Melin-Norkrans agar combined, dilution factor = 10. Controls, agar plates streaked with plain water without bee bread.

Aureobasidium, *Bipolaris*, *Mucor*, *M. sterilia*, *Paecilomyces*, *Rhizopus*, *Scopulariopsis,* and *Trichoderma* were present as irregular, variable components. Bee bread from the organic orchard was distinctive in having significantly higher percentage and greater frequency of *Fusarium* compared to bee bread samples from Lost Hills and the other Turlock site (Tables 3 and 4). Bee bread that was collected from Terra Bella showed *Aspergillus*, *A. niger*, *Penicillium,* and *Rhizopus* as the major isolates, and *Aureobasidium*, *Cladosporium*, *Fusarium*, *Mucor*, *M. sterilia*, *Paecilomyces,* and *Trichoderma* as irregular, variable components. No fungicide residue analysis was available for the Terra Bella samples. Numbers of fungal

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isolates from Lost Hills compared favorably to numbers of isolates from the organic orchard and Terra Bella sites (Tables 3 and 4), and the number of isolates from Turlock was significantly lower than these.

Mycoflora of Bee Bread Fungi From Washington Honey Bee Colonies

Bee bread from Washington contained significantly fewer fungal isolates (Table 5) than the bee bread from Arizona (Table 2, $p < .05$ in each pairwise comparison). The number of isolates in the samples from Washington compared favorably to the low number of isolates that were recovered in the bee bread from California ($p > .05$; Tables 3–5). Differences in Simpson's diversity index values indicate that no two bee colonies were completely alike (Table 5). In the samples from the national forest (EO, Table 5), *Aspergillus* and *Penicillium* were present in all the samples, with *A. niger*, *Cladosporium*, and *Rhizopus* also appearing with fairly regular consistency. *Alternaria*, *A. flavus*, *Fusarium*, *Geotrichum*, *M. sterilia*, *Nigrospora*, and *Trichoderma* were present in mixed amounts as variable components (Table 5). *Aspergillus*, *Cladosporium*, and *Penicillium* were found in all the samples from Yakima (YA; Table 5), *A. niger* and *Rhizopus* were present in most of the samples, and *A. flavus*, *Aureobasidium*, *Fusarium*, *Mucor*, *M. sterilia*, *Paecilomyces*, and *Trichoderma* were present as variable components. Like the samples from California, low numbers of fungal isolates are associated with bee bread having detectable levels of fungicide residues (Figures 1 and 2 and Table 5).

Comparative Observations Across Apiaries

Mean number of total fungal isolates in bee bread varied inversely as a function of the mean amount of fungicide residues that were detected in the bee bread ($y = -4.0x$, $r = .92$) as shown in Figure 3; thus, the more fungicide, the lower is the number of fungal isolates. In contrast, the mean Simpson's diversity index was a positive correlate of the mean amount of fungicide ($y = 0.089x$, $r = .99$). Bee bread samples from ST and OT colonies that showed a high Simpson's diversity index were samples that contained numbers of *Aspergillus* isolates that were not significantly different from the total number of other fungal isolates that were recovered (Tables 3 and 4). In contrast to ST and OT colonies, the EO colonies contained a larger number of *Aspergillus* isolates that were significantly different from the number of isolates of other taxa (Table 5). Because of the high amount of *Aspergillus* in EO colonies, there is a greater probability that when selecting two fungi at random from the EO colonies the two fungi will be *Aspergillus*; thus, the EO colonies are less diverse (Simpson's diversity index is low). Because the amount of *Aspergillus* in ST and OT colonies is lower, there is higher probability that when selecting two fungi from these colonies the two fungi will be different; thus, ST and OT colonies are more diverse (Simpson's diversity index is high) (Tables 4 and 5). Fungal diversity rises with increasing fungicide residue because the amount of *Aspergillus* is diminished more than for the other genera.

DISCUSSION

In this study, fewer fungal isolates were recovered from bee bread sampled from bee colonies that were exposed to fungicide-treated orchards. A fungal decrease occurred and may be traced to a decrease in the presence of fungicide residues. The fact that the number of fungal isolates fell with increasing amount of fungicide residues in the bee bread in a dose-response manner indicates that the fungicide is responsible for the decrease in the amount of fungi in fungicide-contaminated bee bread. Our results indicate that composition of the bee bread mycoflora and fungicide contamination is a product of fungicide application within the bee's foraging area (2–4 miles; Morse, 1984). One of the main features of this study is the evidence that concentrations of fungicides that are being applied in the field are sufficiently high to

TABLE 5. Bee Bread Mycoflora From Select Washington Colonies

	Number of isolates (%) from bee bread from Washington colonies:								
Fungi	EO1	EO3	EO4	EO6	YA2	YA3	YA6	YA8	Controls
Alternaria	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Aspergillus	3 (27)	1 (11)	2 (13)	2 (20)	3 (25)	2 (17)	4 (29)	2 (25)	0 (0)
Aspergillus flavus	1 (9)	0 (0)	0 (0)	1 (10)	0 (0)	2 (17)	0 (0)	0 (0)	0 (0)
Aspergillus niger	3 (27)	2 (22)	0 (0)	1 (10)	4 (33)	0 (0)	1 (7)	1 (13)	0 (0)
Aureobasidium	0 (0)	0 (0)	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)
Cladosporium	0 (0)	1 (11)	2 (13)	1 (10)	2 (17)	2 (17)	1 (7)	1 (13)	0 (0)
Fusarium	0 (0)	1 (11)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)
Geotrichum	0 (0)	1 (11)	1 (7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Mucor	0 (0)	0 (0)	2 (13)	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)
Mycelia sterilia	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)	1 (8)	0 (0)	1 (13)	0 (0)
Paecilomyces	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)
Penicillium	4 (36)	2 (22)	3 (20)	1 (10)	2 (17)	3 (25)	3 (21)	2 (25)	0 (0)
Nigrospora	0 (0)	0 (0)	1 (7)	1 (10)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Rhizopus	0 (0)	1 (11)	2 (13)	2 (20)	0 (0)	1 (8)	2 (14)	1 (13)	1 (100)
Trichoderma	0 (0)	0 (0)	1 (7)	0 (0)	0 (0)	1 (8)	0 (0)	0 (0)	0 (0)
Total isolates	11	9	15	10	12	12	14	8	1
Simpson index	0.19	0.32	0.56	0.37	0.37	0.63	0.76	0.86	
SE	0.012	0.018	0.015	0.021	0.024	0.025	0.022	0.027	

Note. Table 1 gives the history of each apiary. Number of isolates is pooled from potato dextrose agar, modified potato dextrose agar (pH 5.5), and modified Melin-Norkrans agar, dilution factor = 10. Controls were streaked with water that did not contain bee bread.

get into the bee colony by way of pollen (and subsequently the bee bread). The amounts of fungicides that are being applied in the field are sufficient to reduce the amount of bee bread fungi.

Timing fungicide application in the field before bloom appears, a common safeguard to minimize fungicide contamination in bee colonies exerts little, if any, effect. Bee bread from colonies where delayed spraying occurred such as Lost Hills displayed fungicide contamination and few fungal isolates compared to directly sprayed orchards. The fact that there were more isolates in Arizona colonies (with no detectable fungicide residues) shows that the bee bread mycoflora was not disrupted. In addition, colonies placed in orchards that were not directly sprayed were not safe from fungicide contamination. This was demonstrated in the Turlock orchards, where one organic and one directly sprayed orchard were within a few kilometers of each other. Each orchard produced bee bread that showed nearly identical amounts and types of a variety of fungicide residues, along with a similarity in reduction of fungal isolates and bee bread mycoflora composition. However, the organic orchard had not been sprayed directly, but the bees were foraging in adjacent, sprayed orchards. In fact, the organic orchard showed the highest levels of fungicides because of the surrounding sprayed orchards that were within the bee's flight range. Mullin et al. (2010) noted that fungicide contamination of bee bread might also occur when fungicides are sprayed in evening hours when bees are not foraging, because of proximity to sprayed orchards.

It is important to note that colonies were active and thriving during this time. Bee bread that was nearly devoid of fungi (Turlock samples) originated from active colonies. Bees appear to be capable of managing the effect of fungicide contamination at the level of the concentrations that are applied in the field in our study sites. While colonies were tracked for several months as they were moved, the fate of these colonies at the end of the year was not known, although the beekeeper did report a 59% loss in all his colonies in the spring of 2011 (Bach 2013). The regular, expected loss is 15–25% per year (D. Sammataro, USDA-ARS, Tucson, AZ, unpublished observations, 2013).

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The implication is that the amount and types of fungi that remain in the fungicide-contaminated bee bread must still be capable of adequately fermenting the stored pollen into bee bread because the colony is still active; that is, the bee bread is viable, at least for a short period of time. However, the long-term effects and accumulation of these fungicides in beeswax were not recorded. Other factors also exert an effect on colonies, such as the lack of diversity in their forage (Naug, 2009) and genetic diversity of their queen (Mattila et al., 2012). During the course of the year, bees also are in fungicide-free zones. In addition, other pathogens and parasites cause colonies to perish. These factors were not tracked.

It is important to recognize Martha Gilliam's observations made almost 25 years ago suggesting that beneficial fungi, *Aspergillus*, *Penicillium*, *Rhizopus*, and *Cladosporium*, have a controlling effect on regulating the incidence of chalkbrood in bee colonies (Gilliam, 1979; Gilliam et al., 1988; 1989; highlighted by Wood, 1998; Aronstein and Murray, 2010). Applying Gilliam's work, some beekeepers reported a high incidence of chalkbrood in commercial bee colonies. Data suggest one cause could be that the amount of beneficial fungi was reduced (Gilliam, 1979; Gilliam et al., 1989), particularly *Aspergillus,* as indicated in our study. Studies conducted by Gilliam et al. (1988), identifying that some fungi were beneficial and controlled some pathogenic species, suggest that a large amount of fungi seems to be more important for colony health.

Generally, the fungal composition of bee bread can be summarized as large proportions of *Aspergillus* and *Penicillium*, frequently along with *Cladosporium* and *Rhizopus* appearing as regular isolates, with variation in the minor components. *Mycelium sterilia* also appears as a frequent isolate, because of unchanging conditions of the bee colony environment including 30–35°C, 60–70% relative humidity (RH) between frames, 75–80% RH inside a capped brood cell (Cooper, 1980; Chiesa et al., 1989), and darkness (Chapman, 1993). Consistency in mycoflora with regard to dominant fungal components is anticipated in any habitat wherever bee colonies are placed.

All of the fungi identified and cultured are common bee colony fungi (Gilliam et al., 1989; Gilliam, 1997; Osintseva and Chekryga, 2008) that are brought into the colony by bees as they collect pollen. None of the fungi were unusual. These are fungi commonly found in soil and plant settings (Jennings and Lysek, 1999), so it was not surprising to find them in bee bread, because bee bread originates from pollen. However, a survey of existing, naturally occurring fungi was only available in the Arizona colonies during the course of the collection trips. It was not known which fungi were present in the orchards before fungicide applications were applied. The presence of additional fungi in bee bread is highly probable, because not all fungi can be cultured using these methods (West et al., 2007). The groups of fungi seen in bee bread are perpetuated through fungus–fungus competitive interactions, with the major isolates (*Aspergillus, Penicillium, Rhizopus, Cladosporium*) predominating (Yoder et al., 2012b). This study shows that a decrease in fungal components indicates one condition when the level of fungal components is disrupted when there is fungicide contamination. Some fungicides are fungicidal (kill) and some are fungistatic (reduce growth), depending on the kind of fungus, with each responding differently (Yoder et al., 2012a). The type of fungicide applied, concentration, persistence in the environment, mode of action, and amount carried back into the colony likely produce variable effects in the bee bread mycoflora, and all vary depending on different sites and kinds of forage where bees collect pollen. Moving bee colonies frequently would seem to intensify the negative effects of fungicides on colony health because moving exposes the bees to multiple foraging (and possible contaminated) habitats. It also exposes colonies to other beekeeping operations that may have other bee pathogens and pests.

Aspergillus flavus was also found in the bee bread samples, which is one of several causative agents of stonebrood disease (Gilliam

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and Vandenberg, 1997). Stonebrood affects bee brood very similarly to chalkbrood in that this constituent kills and mummifies the larvae. The mummy is the dried, hardened, typically white, fungal mycelium that takes over the dead brood. The presence of *A. flavus* may be a contributing factor to some otherwise unidentified bee mortality. For inactive colonies where the bees died or left the colony, the bee bread fungus composition profile was similar to that of active colonies, by showing low numbers of fungal isolates and a particularly negative effect on *Aspergillus*.

There could also be an interaction between the fungicides bees are exposed to and other pesticides that beekeepers use for mite control, as well as other compounds that are in the wax (Johnson et al., 2010). The long-term effects of fungicides on bee colonies, if any, are not known at this time. In our study, given the types of fungicides that were sprayed at the approved concentrations that were applied in the field, as long as the bees were provided the opportunity to recover, and perhaps given supplemental food, the colonies could recover. More long-term studies are needed. One of the next steps would be identification of these fungal components to species level.

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