Research Article

Honey Bee Health in Maine Wild Blueberry Production.

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Simple Summary: Wild blueberry is an important North American native crop that requires insect pollination. Migratory western honey bee colonies constitute the majority of commercial bees brought into Maine for pollination of wild blueberry. Currently many stressors impact the western honey bee in the U.S. We designed a two-year monitoring study (2014 and 2015) to assess the potential health of honey bee colonies hired for pollination services in wild blueberry fields. We monitored the colony health of 9 hive locations (3 hives / location) in 2014 and 9 locations (5 hives / location) in 2015 during bloom (May–June). Queen health status, colony population size, rate of population increase, and pesticide residues on pollen, wax, and honey bee workers were measured during bloom. In addition, each hive was sampled to assess levels of mite parasites, viruses, and Microsporidian and Trypanosome pathogens. Different patterns in colony health were observed over the two years. Factors predicting colony growth rate in both years were Varroa mite infestation and risk due to pollen pesticide residues during bloom. In addition, recently discovered parasites and pathogens were already observed in most these colonies suggesting that parasites and diseases spread rapidly and become established quickly in commercial honey bee colonies.

Abstract: A two-year study was conducted in Maine wild blueberry fields (*Vaccinium angustifolium* Aiton) on the health of migratory honey bee colonies in 2014 and 2015. In each year 3-5 colonies were monitored at each of 9 wild blueberry field locations during bloom (mid-May until mid-June). Colony health was measured by assessing percent worker and sealed brood rate of change from the beginning of bloom until the end of bloom. Potential factors that might affect colony health were queen failure or supersedure; pesticide residues on trapped pollen, wax comb, and bee bread; and parasites and pathogens. We found that Varroa mite and pesticide residues on trapped pollen were significant predictors of colony health as measured by the percent rate of change of sealed brood during bloom. These two factors explained 71% of the variance in colony health over the two years. Pesticide exposure was different in each year as were pathogen prevalence and incidence. We detected high prevalence and abundance of two recently discovered pathogens and one recently discovered parasite, the trypanosome *Lotmaria passim* Schwartz, the Sinai virus, and the phorid fly, *Apocephalus borealis* Brues.

Keywords: Varroa mite, pesticide residues, pathogens, *Apis mellifera*, migratory hives, Sinai virus, *Lotmaria passim*, *Apocephalus borealis*

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1. Introduction

Wild blueberries are a northeastern North American native crop that is primarily produced in Maine, the Canadian Maritimes and Quebec along with limited crop land area in New Hampshire, Massachusetts, and Michigan [1]. The crop is not planted and consists of several wild sympatric *Vaccinium* species along with a hybrid. When forest is clear-cut, these native understory species flower and produce fruit [1]. In Maine, the most abundant species is *Vaccinium angustifolium* Aiton, but other species are: *V. myrtilloides* (Michx.), *V. boreale* I.V. Hall and Alders, *V. pallidum* Aiton, and the hybrid: *V. angustofloium* x *V. corymbosum* [2]. Maine accounts for 97% of the total U.S. wild blueberry production [3].

Wild blueberries are 100% dependent upon animal pollinators, with bees considered the most important pollinators [4,5,6]. There are more than 120 native bee species associated with wild blueberry in Maine [7]. Native bees that have been studied are highly efficient and effective pollinators in terms of per visit pollen deposited on stigmas, short flower handling time [8,9], and spatial pattern foraging behavior [10]. Commercial bumble bees, *Bombus impatiens* (Say) are also highly effective pollinators of wild blueberry [11,12].

Honey bees, on a per bee basis, are not particularly efficient pollinators of wild blueberry [5,9], but they are effective pollinators. Their effectiveness is due to the high densities of colonies deployed in wild blueberry fields [5], up to 20 hives per hectare with a recommended hive stocking density of 5-10 hives per hectare, depending upon the native bee community abundance [13]. In 2016, more than 80,000 hives were brought into Maine for wild blueberry pollination [Lund, unpublished data]. Although native bees provide about 20-30% of fruit set [5,14], wild blueberry growers rely heavily on honey bees to mitigate the risk associated with native bee community fluctuations in abundance from year to year [15].

The dependence on honey bees by growers for pollination is not without risk. Cold temperatures during bloom in Maine can decrease foraging activity [9,10]. Abundance of flowering plant species in or outside of wild blueberry fields can decrease visitation to wild blueberry [9,12,16]. In addition, unhealthy colonies can exhibit compromised foraging or collapse,[17]. Honey bee colony health can be affected by parasites and pathogens, transportation stress, suboptimal nutrition, low genetic diversity, inadequate queen mating, and pesticide exposure [18-23]. Beekeepers throughout the world have been struggling to keep honey bee colonies alive since major declines and collapses were noted in the early part of this century with the occurrence of Colony Collapse Disorder (CCD) [24]. Colony loss since this time has been demonstrated to be a multiple stressor phenomenon involving the interaction of many factors [25].

Objectives. Not many studies investigating honey bee colony health have addressed colony status on an early spring berry crop during pollination. The objective of this study was to assess the potential health of migratory honey bee colonies brought into Maine wild blueberry fields to provide pollination services during bloom in 2014 and 2015. To determine colony health, colonies were sampled to estimate queen status (presence of queen and oviposition), preparation of queen supersedure, worker population size,

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worker population rate of increase, sealed brood population size, sealed brood population rate of increase, pathogen and parasite loads, and pesticide residues in trapped pollen, wax comb in the brood area, and stored bee bread.

2. Materials and Methods

Study site and colony health measures. This study was conducted in the major wild blueberry growing regions in Maine, Hancock and Washington counties, in the towns of Alexander, Aurora, Cherryfield, Columbia, Deblois, Jonesboro, and unorganized township T22, during the years 2014 and 2015. Beekeepers and blueberry growers were contacted for permission to sample hives in the field during the period of bloom (usually mid-May to mid-June). Several hive locations in wild blueberry fields were monitored each year. We monitored 9 hive locations (fields) (3 hives/location) in 2014 and 9 locations (5 hives/location) in 2015. In each field, hives were on wooden pallets with 4-6 hives per pallet and the numbers of hives per field ranged from 60–200. Colony sampling was conducted three times during bloom. In 2014, bloom was from 18 May–13 June and in 2015 bloom was from 20 May–16 June. Colony health was measured by sampling queen presence, egg laying and the presence of presence of supersedure cells, indicating preparation of queen supersedure. Sealed brood population was calculated by determining the percent area of wax comb on varying sized hive bodies with sealed brood and then converting to numbers of brood using published formulae [26]) Worker population size was calculated by determining the % area of wax comb on varying sized hive bodies with workers and then converted to numbers of workers using published formulae [26]). The health status sampling was performed twice, shortly after hive deployments in blueberry fields and just before bloom ended. A measure of population growth was calculated by determining the % rate of change from the first sample to the second sample of both sealed brood and workers (see equation 1). Previous studies have found that colony population size and rate of change is a reliable measure of colony health [27].

$$\% \text{ rate of change} = \frac{(\text{population size at time t} - \text{population size at time t+1})}{(\text{population size at time t})} * 100 \quad [1]$$

Pesticide residues. In both 2014 and 2015 pesticide residue analysis was conducted on trapped pollen sampled during peak bloom on 3 colonies/location. Samples were pooled by location in each year. Front entrance pollen traps (Anatomic Front Mount Pollen Trap®) were attached to the entrances without the trapping gate set for 24 hours, allowing colonies to adapt to the trap. On the following day the trapping gate was closed and pollen was trapped for 48 hr [28]. Wax comb (ca. 100 cm^2) was collected adjacent to the brood area of 3 hives/location during late bloom and pooled by location. In 2015 only, 5-10 gm/hive of bee bread (stored pollen mixed with nectar) was extracted from wax comb above the brood area, from 3 hives/location and pooled by location. Residue samples were transported at the end of the day of collection from the field to the laboratory in Orono, ME in insulated coolers containing ice-packs. Once at the laboratory samples were stored at -80 ºC. Samples were shipped overnight to the pesticide residue analysis laboratories when requested. In 2014 pesticide analyses of samples were conducted at the Connecticut

Agricultural Experiment Station, New Haven, CT., U.S. and in 2015 the analyses were conducted by the USDA National Science Laboratory, Gaston, N.C., U.S. The Connecticut Agricultural Experiment Station analytical chemistry laboratory used high pressure liquid chromatography analysis targeting 140 different pesticides and metabolites after extracting the residue targets using a modified QuECHERs procedure [29]. More details of the procedures can be found in Ostiguy et al. [30]. The USDA National Science Laboratories in Gastonia, NC. Screened for 200 agricultural pesticides and metabolites used gas chromatography and high-pressure liquid chromatography with mass spectrometry. This laboratory also utilized a modified QuECHERs procedure for extracting residue targets from the various matrices. Limits of detection (LOD) mostly ranged from 5 – 25 ppb (maximum = 50 ppb) depending upon the matrix that residues were extracted from and the specific pesticide (eg. pyrethrin LOD = 50 ppb). Details of the procedures used at the National Science Laboratories can be obtained by contacting Dr. Jonathan Barber, Chemistry section supervisor (Jonathan.Barber@usda.gov).

Limits of detection (LOD) ranged between 0.5 and 20 ppb depending upon the matrix (pollen, wax, bee bread) that residues were extracted from. Most of the compounds had an LOD of less than 5ppb with 88 compounds at 1 ppb or less. However, the pesticides in the two laboratories' screens differed and the detection levels differed for many of the same pesticides that were in the screen. Because of this we chose to minimize laboratory bias by only selecting pesticides that both laboratories searched for and we only considered pesticide detections in trapped pollen and wax comb in 2014 and 2015 when we applied the higher level of detection (LOD) to both years of analytical results. For example, if the insecticide Phosmet in pollen had an LOD of 1 ppb by one laboratory, but 10 ppb by the other laboratory, we only considered detections for Phosmet in both 2014 and 2015 when concentrations were at 10 ppb or higher. We realize that this reduced the number of pesticide detections and the overall total concentration of residues by eliminating detection of low concentrations over the two-year period, but it provided a consistent benchmark for making comparisons of exposure and toxic risk between years. We also corrected the detections as described above for bee bread even though this matrix was only sampled in 2015.

A quantitative measure of pesticide risk was calculated from the residue data to determine if levels of exposure observed from pollen, wax, and bee bread affected colony health. Contact Hazard Quotients were calculated using methods by previous authors [28,30], but because Oral LD50 estimates are: 1) less available, and 2) because contact and oral Hazard Quotients are highly correlated [28]; we only estimated contact Hazard Quotients for this study. To calculate the contact Hazard Quotient, lethal dose 50th percentile values (LD50 in units of ppb) were compiled for all detected compounds based upon available literature and public databases (see [28,30] for information on databases). We used the LD50 values of parent pesticide compounds if LD50s were not available for metabolites [28,30]. Then we divided the concentration of each pesticide quantified in trapped pollen, wax comb, or bee bread for a given location by the contact LD50 estimated for honeybees. Contact LD50 values reported in terms of µg/bee were converted to ppb

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relative to body weight (ng pesticide per g bee) by multiplying each value by a factor of 10,000; this is an approximate equivalent to 1,000 ng per µg÷mean bee weight of 0.1 g [32]. An estimated Hazard Quotient of 1.0 suggests that the exposure level by contact will result in 50% mortality to colony populations. A Hazard Quotient greater than 1.0 represents an expectation of high proportions of mortality. Based upon these Hazard Quotients, we assessed risk both at the individual pesticide compound level, pesticide use-group level, and also additively across all pesticides detected, which provides a measure of total colony risk. This total colony Hazard Quotient assumes that effects due to pesticides are additive and this is most likely not the case based upon studies showing synergy among pesticides in honey bees [33,34]. However, we feel that our use of an additive Hazard Quotient is acceptable because we use total colony risk only as a relative measure of colony stress for comparing locations and years and not as an absolute estimate of acute mortality.

Parasites and Pathogens. Varroa mite, *Varroa destructor* (Anderson and Trueman), and tracheal mite, *Acarapis woodi* (Rennie), infestations were sampled during peak bloom by collecting ca. 400 workers (collected in 150 ml urine cups), ca. 200 nurse bees from the brood area and ca. 200 older field bees from honey and pollen frames in each of 3 (2014) or 5 (2015) hives at each location. Bees were preserved in ETOH until processing. In the lab, samples were counted, Varroa mite infestation was quantified using modified methods by Hendrickson [35]and then dissected for tracheal mite assessment using the technique developed by Sammataro [36]. Varroa mite was quantified as the number of mites per 100 worker honey bees and tracheal mite was quantified as percent honey bee worker infestation. During the dissections for tracheal mite, we also looked for the parasitic phorid fly or "zombie fly", *Apocephalus borealis* Brues, and quantified prevalence as percent bees with phorid parasites.

During late bloom a sample of 200 workers from each hive at each location and year was taken from both brood and honey comb to assess common honey bee viruses and other pathogens. Honey bees were transported at the end of the day of collection from the field to the laboratory at the University of Maine, Orono, ME in insulated coolers containing ice-packs. Once at the laboratory, samples were stored at -80 ºC. In 2014 bees were shipped on dry ice to the National Honey Bee laboratory in Beltsville, MD USA for molecular detection of pathogens using qRT-PCR analysis. In 2015 samples were shipped on dry ice to the North Carolina State University Apiculture Queen Disease Clinic, NC USA for molecular detection of pathogens, again using qRT-PCR analysis. Each sample was screened for 6 known honey bee viruses in 2014: Israeli Acute Paralysis Virus (IAPV), Black Queen Cell Virus (BQCV), Kashmir Bee Virus (KBV), Chronic Bee Paralysis Virus (CBPV), Sacbrood Virus (SBV), and Deformed Wing Virus (DWV); and eight known honey bee viruses in 2015: Acute Bee Paralysis Virus (ABPV), Chronic Bee Paralysis Virus (CBPV), Black Queen Cell Virus (BQCV), Deformed Wing Virus strains A (DWVA) and B (DWVB), Israeli acute paralysis virus (IAPV), Sacbrood Virus (SBV), and Lake Sinai Virus (LSV). In 2014 one microsporidian pathogen – *Nosema ceranae* (Fries et al., 1996) and the Trypanosome group of pathogens at the order level

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was screened. In 2015 *Nosema* (at the genus level), two Trypanosome pathogen groups, *Crithidia* (at the genus level), and other Trypanosomes not including *Crithidia* (at the order level). This Trypanosome was later determined to be only one species, *Lotmaria passim* Schwartz (Kinetoplastea). At both testing facilities, RNA was quantified on a Nanodrop Spectrophotometer, diluted, and then total cDNA was synthesized and used in a PCR reaction. The Normalization of final values to two reference genes (Ancr1 and AB Actin) values was performed in GeNorm [37] at the National Honey bee laboratory and the genes (Apo28s, CamllK) were used for normalization at the NC laboratory. At both facilities Quantitative PCR was used to assess not only presence of the pathogens, but also the intensity of infection (estimated number of viral copies). The normalized intensity of infection was logarithm (base 10) transformed for all analyses.

Statistical analysis. Graphical visualization was used to illustrate observed colony health metrics and potential causal factors of colony health. Linear correlation (Pearson) was used to determine positive or negative associations between pesticide residues (log(ppb)) in the three matrices (trapped pollen, wax comb, and bee bread) in each year. Non-parametric correlation (Spearman) was used to asses if the different sampled matrices shared the same pesticide residues (presence/absence). Correlation (Pearson) was also used to determine if associations existed between parasite and pathogen incidences. These analyses were only conducted for parasites and pathogens screened in both years. In these correlations for DWV as a variate, the DWV marker in 2014 was used with the DWV-A strain for 2015. Mixed models were used to test the effect of year on colony percent rate of change, pesticide residues, parasites, and pathogens. Year was a fixed effect and the residuals were assumed to have a random correlation structure within year. A mixed model was also used to determine what potential causal factors determined colony health. The colony health metric, sealed brood % rate of change was the dependent variable chosen for the analysis. Because sealed brood % rate of change was significantly related to worker % rate of change, we chose only to use one of the measures of colony health, % rate of change of sealed brood. The location-level Hazard Quotients for pollen, wax comb, and both wax and pollen combined, and parasite measures (individual taxa, and all combined as total parasites) were fixed effects. Year and its interactions with fixed effects were included in the models as random effects. Because pesticide analysis was conducted on samples pooled over colonies within a location, the causal factor analysis was performed with location-level data using the pooled and averaged values by locations for all dependent and independent variables. All correlations and mixed models were estimated with JMP statistical software [38].

3. Results

Colony Health. The change in population size was different in 2014 compared to 2015 (Fig. 1A & B). Mean sealed brood rate of change was significantly less in 2014 (-33.8±15.7 (s.e.) %) than 2015 (40.9±3.8%) ($F_{(1,8)}$=19.597, P=0.002). This same pattern was also observed with worker populations, 2014 (-40.2±10.7%) vs 2015 (33.9±4.8%) ($F_{(1,8)}$=37.660, P<0.001). Figure 1A shows that one third of the locations had an in

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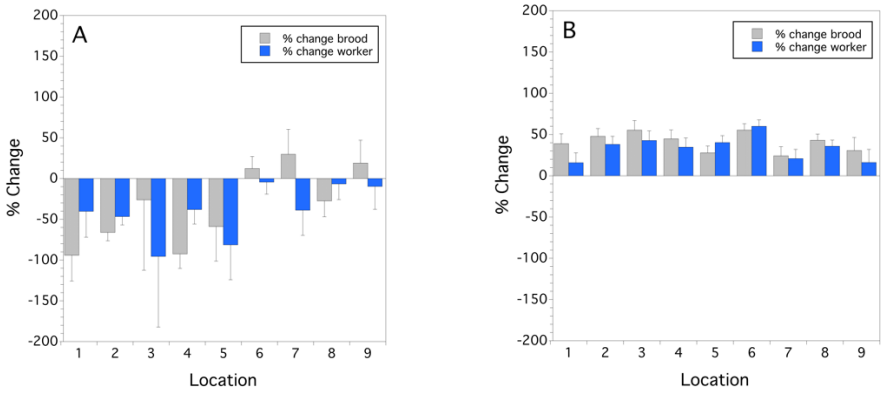

Figure 1. Mean colony population rate of change from initial population size over the bloom period per location for sealed brood and workers for 2014 (A) and 2015 (B), zero on the y-axis represents no change in sealed brood or worker population size during bloom. Error bars are standard errors of the mean (n=3 and n=5, 2014 and 2015; respectively).

crease in colony worker populations over the bloom period in 2014. In contrast, all of the locations in 2015 experienced increase in population size during bloom (Fig. 1B).

The lack of population increase did not appear to be associated with queen supersedure. In 2014, only 11.1% of the colonies sampled had undergone supersedure and in 2015 supersedure occurred in only 6.7% of the colonies sampled. The relationship between the % rate of change in sealed brood and that of workers for all 72 colonies sampled over the two years is shown in Figure 2. We found that the % rate of change of workers determined the rate of change of sealed brood ($F_{(1,68)}$=8.032, P=0.006) and that while year was significant ($F_{(1,68)}$=6.557, P=0.013), there was no year x worker rate of change interaction ($F_{(1,68)}$=0.304, P=0.583). Forty-eight percent of the variation in the % rate of change in sealed brood is explained by the % rate of change in workers (Fig. 2). As the rate of population decline increases (from 0% to -200%), workers decline faster than sealed brood, but when the rate of population change increases (>0%), workers increase at a higher rate than sealed brood (see regression line in Fig. 2).

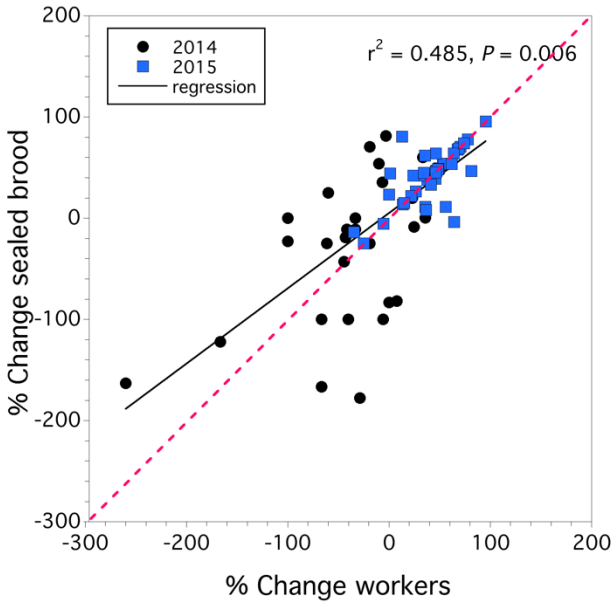

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Figure 2. Linear relationship between % rate of change in workers and % rate of change in sealed brood. Red dashed line is 1:1 slope.

Pesticides. In 2014, 11 pesticides and their metabolites were detected in trapped pollen and 21 compounds were detected in wax comb. In 2015, 7 pesticides and their metabolites were detected in trapped pollen, 13 in wax comb, and 9 in bee bread. The five pesticides or metabolites detected in the highest concentrations in both trapped pollen and wax in 2014 and trapped pollen, wax comb, and bee bread in 2015 are listed in Table 1. Fungicides, herbicides, insecticides, and miticides were represented in the residues with the highest concentrations. The concentrations listed in Table 1 have high variation between hives/location as reflected by the large standard errors relative to the mean concentrations.

Table 1. Pesticide residues in highest average concentrations (ppb/location) in trapped pollen, wax comb, and bee bread in 2014 and 2015.

Year	Trapped pollen (ppb)[1]	Wax comb (ppb)	Bee bread (ppb)
2014	DMPF[2] (3853.7±3262.9) M[3] tau-Fluvalinate (462.3±280.8) M Chlorothalonil metabolite[4] (257.9±118.9) F Boscalid (47.9±81.7) F Propiconazole (24.8±9.9) F	tau-Fluvalinate (3849.0±3756.6) M DMPF[3] (1458.6±1219.4) M Chlorothalonil metabolite[4] (107.4±73.5) F Coumaphos (54.1±29.8) M Propiconazole (54.0±46.9) F	Not sampled in 2014
2015	Fludioxonil (98.8±68.5) F DMPF[3] (95.5±74.0) M Cyprodinil (85.7±60.1) F Phosmet (16.3±12.6) I Sethoxydim (13.3±7.4) H	DMPF[3] (588.9±471.5) M Tebuconazole (6.0±6.0) F Coumaphos (5.9±1.5) M tau-Fluvalinate (5.3±5.3) M Pyrimethanil (2.2±2.2) F	Fludioxonil (621.1±614.9) F Cyprodinil (475.8±468.1) F Phosmet (143.0±114.7) I Chlorothalonil metabolite[4] (117.8±116.5) F Sethoxidym (42.3±39.6) H

[1] Mean ± s.e. concentration (ppb) averaged over location
[2] DMPF (2,4 Dimethylphenyl formamide) is a metabolite of the miticide Amitraz for Varroa control
[3] Use Category: F=fungicide, H=herbicide, I=insecticide, M=miticide for Varroa mite control
[4] 4-hydroxychlorothalonil is a metabolite of Chlorothalonil fungicide

Exposure to pesticides (parent chemical compounds and their metabolites) in trapped pollen was greater in 2014 than in 2015 (Fig. 3A), both in numbers of pesticides/location ($F_{(1,15)}$=7.124, P=0.018) and total residue concentration (ppb)/location ($F_{(1,15)}$=4.892, P=0.043, mixed model with logarithm transformed ppb). Also, the number of pesticides detected in wax comb were greater in 2014 than in 2015 ($F_{(1,14)}$=25.204, P<0.001; Fig. 3B), as was the log concentration (ppb) of pesticides in wax comb higher in 2014 than in 2015 ($F_{(1,14)}$=4.719, P=0.048; Fig. 3B). Trapped pollen, in MOST cases is thought to represent exposure from the flowers that honey bees are foraging on at the time of trapping, but because we found a high level of miticides in the pollen, this assumption has to be questioned and probably represents both pesticide contamination from inside and outside the hive. Pesticides contaminating wax is often thought to be an integration of pesticide exposure over a longer time period such as a growing season or several years until new foundation replaces the older wax comb. Residues in bee bread in 2015 were similar to the

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residues in the 2015 trapped pollen. The pesticide numbers detected in bee bread were 3.0±0.7 and the mean log concentration of residues was 2.1 ± 0.4.

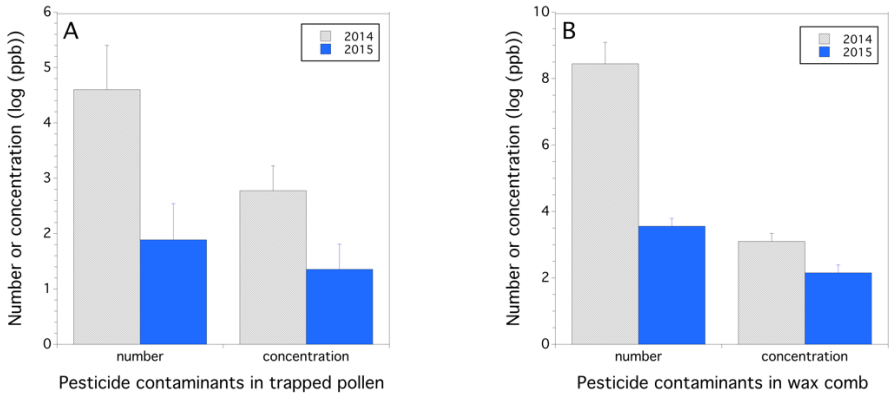

Figure 3. Concentration of pesticides and metabolites and number of pesticides detected per hive location in 2014 and 2015 in trapped pollen (A) and wax comb (B), concentration (ppb) is logarithm base 10 transformed.

All routes of exposure (pollen, wax comb, bee bread) for the four pesticide use groups (fungicides, herbicides, primarily insecticides, and in-hive miticides), as reflected by residue concentration, are compared in Figure 4. In 2014, the pesticide group percent composition in trapped pollen (Fig. 4A) was similar to what was detected in wax comb (Fig. 4B), with majority being miticides used to treat Varroa mite. Miticides comprised 90.2% of the total concentration of detected pesticide residues in trapped pollen and 95.1% in wax comb. However, in 2015, a very different pattern was observed. In 2015, the pesticide group percent composition in trapped pollen was fungicides (56.6%) and to a lesser extent miticides (30.1%) in (Fig. 4C) whereas pesticide detections in wax comb (Fig. 4D) was almost exclusively miticides (96.9%). The bee bread (Fig. 4E) was similar to trapped pollen in 2015 (Fig. 4C), with pesticide detections being primarily comprised of fungicides (85.8%). This data shows that the makeup of contaminants, even at the level of pesticide use group can vary greatly across years in the same crop system and geographic region.

Linear Pearson correlation analysis revealed that in 2014 the log (ppb) levels of pesticide residues in wax and pollen involving all compounds that were detected showed that residue concentrations found in trapped pollen were correlated to residue concentrations in wax comb (r=0.329, P=0.007, n=66). When the presence or absence of detected compounds in wax and pollen were tested for correlation, we also found evidence for a significant correlation (Spearman's ρ=0.305 P=0.013, n=66). In 2015, no correlation was found between trapped pollen and wax comb (P=0.738). However, bee bread was positively correlated with trapped pollen (r=0.0.741, P<0.007, n=40), but negatively correlated with wax comb (r=-0.577, P=0.032, n=42). The presence of specific residues of all the matrices in 2015 were correlated, trapped pollen with wax comb (ρ=0.345 P=0.009, n=56), bee bread negatively correlated with trapped pollen (ρ=-0.577 P<0.001, n=40), and bee bread with wax comb (ρ=0.674 P<0.001, n=42). When both years were considered together, log residue concentrations and presence/absence of pesticide residues were correlated between trapped

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pollen and wax comb (r=0.239, P=0.008, n=122; ρ=0.222, P=0.014, n=122, concentration and presence/absence; respectively).

The honey bee Hazard Quotient (log transformed) varied significantly between the two years for trapped pollen ($F_{(1,15)}$=6.029, P=0.027). The Hazard Quotient of wax comb was only significant at α=0.1 ($F_{(1,16)}$=3.983, P=0.063). Trapped pollen in 2014 had a higher Hazard Quotient than was found in 2015. The average Hazard Quotient for trapped pollen in 2014 vs 2015 was 2.38±2.01 vs 0.06±0.05. For wax comb, the Hazard Quotient was also higher in 2014 than 2015: 2.86±1.94 vs 0.38±0.28. The trapped pollen Hazard Quotient was significantly greater than the wax comb Hazard quotient in 2015 ($F_{(1,8)}$=8.095, P=0.022), but not in 2014 ($F_{(1,8.2)}$=0.311, P=0.592). Bee bread, only sampled in 2015, had a Hazard Quotient

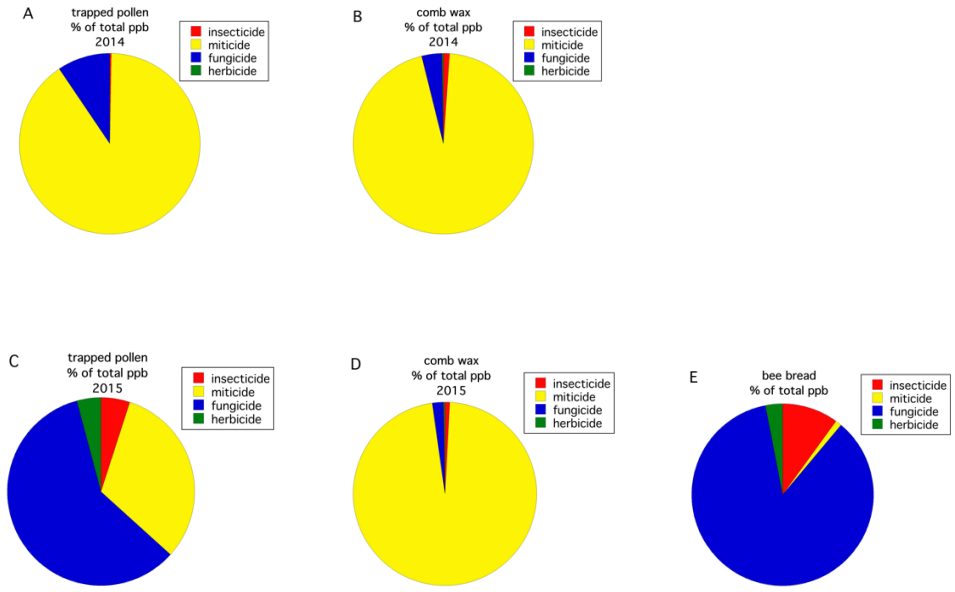

Figure 4. Percent composition of pesticide residue concentration (ppb) by use group in trapped pollen in 2014 (A), wax comb in 2014 (B), trapped pollen in 2015 (C), wax comb in 2015 (D), and bee bread in 2015 (E).

of 0.02±0.01, and was significantly different than the mean Hazard Quotient of wax comb (P=0.011), but not trapped pollen (P=0.926, Tukey HSD multiple comparison). Figure 5 shows the percent composition by pesticide use-group of the calculated Hazard Quotient by year and route of exposure (*ie.* trapped pollen, wax comb, bee bread). For both years the risk to exposure of trapped pollen and wax comb is almost entirely due to miticides (Fig. 5A-D), although insecticides contribute a measurable proportion of the total risk. Insecticides can contribute a disproportional amount of risk relative to concentration. As an example, in 2015, insecticides constituted 0.35% of total pesticide concentration (ppb) in wax comb, but 4.8% of the total Hazard Quotient (Fig. 4D and 5D), and 10.1% of total pesticide concentration in bee bread, but 58.3% of the Hazard Quotient (Fig. 4E & 5E). This is due to the high proportion of total ppb contamination by miticides (Fig. 4A-D). Bee bread in 2015 departs from the pattern of miticides contributing to the majority of the Hazard Quotient with the largest component of risk being insecticides (58.3%, Fig. 5E), but then followed by miticides (38.7%, Fig. 5E).

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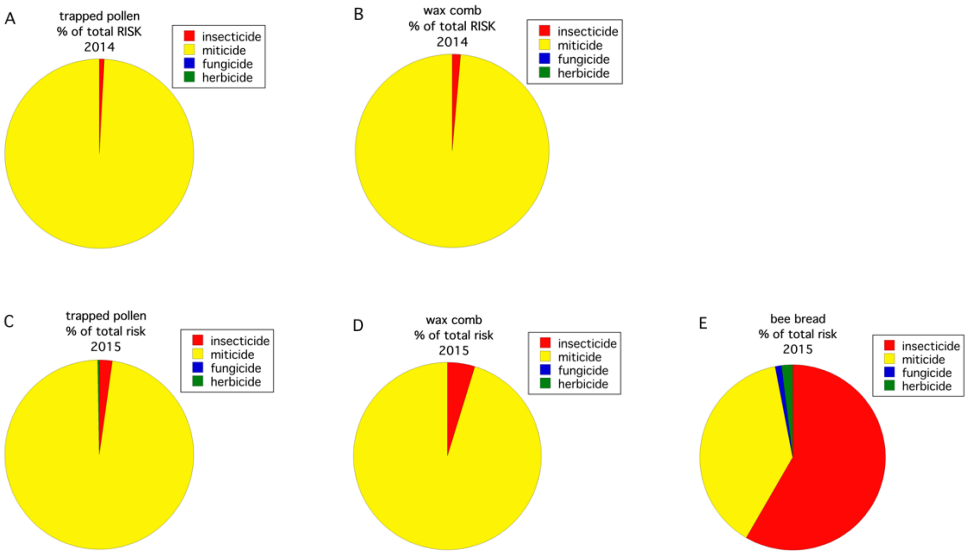

Figure 5. Percent composition of Hazard Quotient by use group in trapped pollen in 2014 (A), wax comb in 2014 (B), trapped pollen in 2015 (C), wax comb in 2015 (D), and bee bread in 2015 (E).

Parasites and pathogens. Varroa mites were found in higher infestation levels in 2014 compared to 2015 ($F_{(1,8)}$=6.509, P=0.034), while tracheal mite infestation was not different between years (P>0.05) (Fig. 6A-B). Phorid fly eggs and larvae (*Apocephalus borealis*) were only found in 2015, but they were common at all locations (mean infestation/location=5.5±1.0%) (Fig. 6B). The green dashed line in Figures 6A and 6B are the treatment threshold commonly recommended for Varroa Mite [39]. In 2014, five of the nine locations had colonies with Varroa mite infestation greater than the threshold of 3 mites/100 honey bees [39]. In 2015, none of the locations exceeded this threshold.

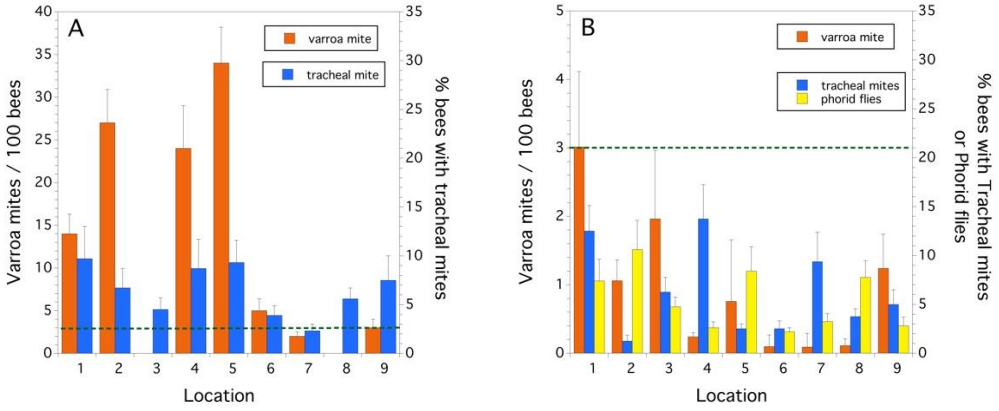

Figure 6. Infestation rates of varroa mite, tracheal mite, and phorid flies in 2014 (A) and 2015 (B). Error bars are standard errors.

In 2014, BQCV, DWV, and *N. ceranae* were detected at all nine locations, but virus incidences were at low to moderate levels relative to *L. passim* (Fig. 7A)). Sac brood virus and Trypanosome infections were detected at all but one location in 2014 (Fig 7A). Three viruses (KBV, IAPV, CBPV) were either at an extremely low prevalence and incidence or

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were absent in the honey bee populations we sampled. Because of this we did not plot these three viruses (Fig. 7A). In 2015, several viruses (ABPV, CPBV, and IAPV) were either not detected or were very low in prevalence and incidence (not plotted). Figure 7B

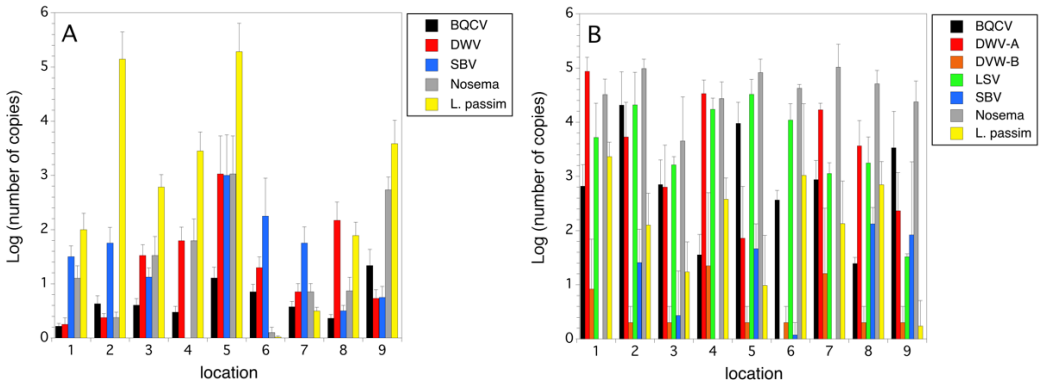

Figure 7. Pathogen incidence (logarithm base 10 (number of copies)) by hive location in 2014 (A) and 2015 (B). Error bars are standard errors.

shows that in 2015, five viruses and two unicellular pathogens were present at most of the hive locations. DWV-A, LSV, and *Nosema* were the pathogens with highest incidence. The *Nosema* genus level markers most likely represented either *N. ceranae* or *Nosema apis* (Zander, 1909). In both years *L. passim*, a low-level trypanosome pathogen, was quite common and had fairly high incidence. When linear correlation analysis was performed between all log transformed copy numbers for pathogen markers in both 2014 and 2015, we found 3 significant (Bonferroni corrected) associations in honey bees. Prevalence was high among all the commonly detected pathogens and so co-association within a colony was common. There were significant correlations between incidence of *Nosema* and BQCV (r=0.682, P<0.0001); and *L. passim* and BQCV (r= -0.351, P=0.001) and *L. passim* and DWV (r=0.339, P=0.001).

We also found a causal relationship between the square root of Varroa mite density (mites/100 bees) and the logarithm transformed number of copies of the DWV marker (a proxy for disease intensity) ($F_{(1,73)}$ =13.883, P<0.001), but there was also a significant year x sqrt (Varroa mite) interaction ($F_{(1,73)}$ =4.656, P=0.034). The overall model was significant ($F_{(3,75)}$ =4.880, P=0.004), r^2 = 0.133 (Fig. 8). The slope was significantly lower in 2014 (β=0.328±0.071) than in 2015 (β=1.229±0.46).

Causal effects of colony health. Because % change in sealed brood populations explained 48% of the variation in percent change in worker populations in a colony, we chose to model only percent change in sealed brood as a proxy for colony health. The percent change in sealed brood was best explained by a negative effect of the logarithm of Varroa mite density (β= -78.128±17.076, $F_{(1,14)}$=20.933, P<0.001) and a negative effect of the logarithm of trapped pollen Hazard Quotient (β= -21.096±9.118, $F_{(1,14)}$=5.353, P=0.036). Varroa mite density and trapped pollen Risk Quotient were independent, not autocorrelated (P=0.610). Scaled estimates of slopes suggest that Varroa mite is 1.7 times more influential

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in affecting % change in sealed brood than the Hazard Quotient of trapped pollen. Other factors, on an individual basis, also determined % sealed brood, but they were either not

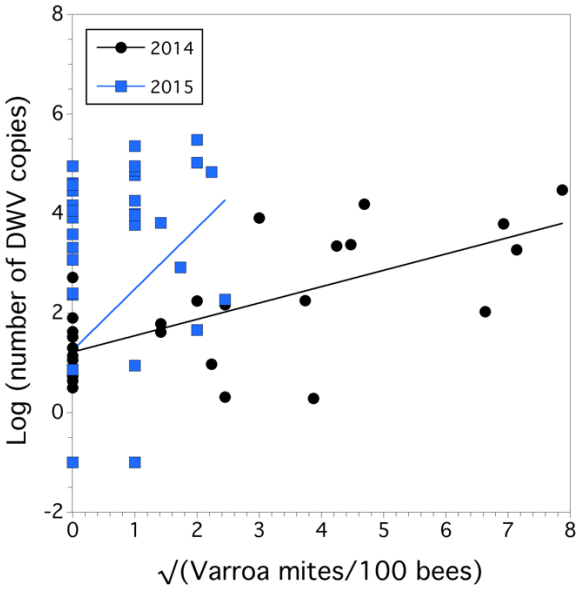

Figure 8. Relationship between Varroa mite density (sqrt transformed) and the intensity of DWV infection (logarithm base10 (number of DVW copies) for each year.

significant (P>0.05), or they were correlated with the causal factors that were the best predictors (e.g., log (number of DWV copies) is determined by Varroa mite, as previously shown). The proportion of variance explained in % change in sealed brood by the model was high (conditional r^2=0.578). The Hazard Quotient for wax comb was not a predictor of colony health, and nor were any of the pathogens. When varroa mite was not put in the model, tracheal mite infestation demonstrated a trend toward being a significant predictor of negative colony health (P=0.148), although tracheal mite infestation and Varroa mite density were not correlated with each other (P=0.159). *Nosema* was positively associated with % change in sealed brood (r=0.651, P=0.004), but we viewed an increase in *Nosema* as a result of an increase in colony population size and not a causal factor of colony percent change in sealed brood because it did not support current knowledge about *Nosema* in that infection does not increase colony health and so it was not included in a model to determine causality of colony health.

4. Discussion

Wild blueberry is an obligate insect pollinated plant, mostly dependent upon bees [5,40]. Migratory honey bee hives are used to supplement the pollination by native bee species [5,41]. There has been concern by both honey bee keepers and wild blueberry growers about the health of colonies that are brought in for pollination services each year. In our study we found that colony health varies among years.

In 2014 colony population size declined, over the bloom period. In 2015 colonies increased in population size during bloom. Although the plants resupply nectary tissues daily during bloom, wild blueberry can exhibit nectar dearth during bloom, [42]. Most

migratory colonies brought to wild blueberry for pollination are fed sugar syrup during bloom and so we suspect that differences we observed between years was not due to starvation. Nutritional content of wild blueberry has been shown to be suboptimal for honey bees [43], but honey bees usually do not collect a high percentage of blueberry pollen while foraging and most of their dietary intake of pollen comes from other plant species surrounding wild blueberry fields [12]. We speculate that the nutritional quality of pollen would not fluctuate annually in a way that would explain the observed differences in colony health between 2014 and 2015.

We found that colony health over the two-year study period was best described by Varroa mite densities and the Hazard Quotient estimated from trapped pollen pesticide residues (both logarithmically transformed). The residue concentrations in 2014 were higher than 2015, but in both years the amount of miticide and miticide metabolites found in both pollen and wax comb was high. In this study we have assumed that Amitraz, Coumaphos, Fluvalinate, and their metabolites were all due to the use of these compounds as miticides to control Varroa mite. This is based upon the supposition that all of formulations of these compounds are currently registered for Varroa mite control but also because none of these pesticides were or are recommended for use in wild blueberry insect pest management [44] and as far as we know they have never been used by wild blueberry growers for crop pest management (Drummond pers. com.). The dominance of miticides in pollen and wax comb has been reported in a large-scale apiary study conducted in Spain [45]. These miticides are toxic to honey bees at high doses which were detected in this study [30]. In 2014, miticide levels in hives were exceptionally high and yet Varroa mite levels were also extremely high. This situation probably reflects Varroa mite resistance to Amitraz and Coumaphos miticides in the U.S. Resistance was first observed for both miticides in the late 1990s and early 2000s [46,47], although it can be seen that a decade and a half after the first reports of resistance, these miticides were still being heavily used.

Even though we found Varroa mite and the trapped pollen residue Hazard Quotient to be significant predictors of % colony population change during bloom one must still be cautious in concluding that the cause of the differences in colony health was due to only these two factors. Pesticide residues in the hive result in a complex dynamic and one measure, trapped pollen Hazard Quotient, may not adequately capture the mechanisms at play and subsequent health risk to the colony. We are aware that our measure of risk to honey bee colonies is crude. We only estimate contact risk based upon the LD_{50} response to pesticides in workers. Our approach did not capture toxicities of "inert" ingredients used in pesticide formulations which have been shown to have detrimental behavioral and physiological effects on honey bees [48]. It also did not capture the oral risk which can't be predicted from contact risk [30], or larval sensitivity to pesticide exposure [49], and synergy among mixtures of pesticides which will be the norm in the hive environment [50].

It is difficult to make predictions of the environment outside the hive with trapped pollen. Our initial assumption, along with other authors of several published studies [51], is that trapped pollen represents the current contamination of pollen and floral surfaces in

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the foraging territory of the honey bee colony (in this study, blueberry fields). However, this might not be a valid assumption for all residues detected. We are suspicious that the high level of miticides that would be used for Varroa mite control detected in pollen would be an independent measure of floral contamination. Although, it could be the case that honey bee body surfaces contaminated with miticide after a recent miticide treatment contaminated pollen by direct body contact of the contaminated honey bees with floral surfaces or that contamination of pollen occurred when previously contaminated honey bees groomed the pollen off of their bodies and packed the pollen in their corbiculae [52]. Bee body contact with floral surfaces is the suggested mechanism of transfer of honey bee parasites and pathogens to native bees [53].

Wax comb residues were initially assumed to be predictive of colony health since they represent the integral of incoming contamination over time (minus degradation). However, in our study, trapped pollen Hazard Quotients was a better predictor of colony health. This may be explained by difference in actual exposure (food vs contact through comb) which is difficult to measure but has been demonstrated with differences in outcomes to queens exposed to different sources of pesticides during development [54]. In addition, Hazard Quotients of trapped pollen and wax comb were not correlated (P=0.429). The Hazard Quotients of bee bread, the processed food of larvae, was also expected to be a good predictor of colony health, especially sealed brood percent change, but we only had data from 2015 and so this metric could not be adequately tested. However, the Hazard Quotients of bee bread was significantly correlated with the Hazard Quotients of trapped pollen in 2015 (r=0.879, P=0.002) and so the risk due to contaminated bee bread may also be a good predictor of colony health in future studies.

Pesticide residues can have acute effects on individual honey bees and colony populations [55] or exposure can result in more chronic conditions [56]. Symptoms of pesticide exposure can be death of individuals in the colony [55], reduction in colony growth rate [57] reduction in queen productivity, increase in supersedure or queen loss [54], reduction in cognition and sensory modalities [58], and repellency of floral resources to foragers [59].

While colony losses have been shown to be caused by exposure to pesticides, especially insecticides, fewer studies have shown that miticides used to treat Varroa mite can have negative effects on colony population size. Johnson et al. [60] showed that interactions between miticides can result in highly toxic responses in honey bee workers. The use of Hazard Quotients has been used to assess exposure and potential colony effects [30,31], but a few studies have used these metrics with success to explain colony losses or declines in colony population size over time [61,62].

Honey bees in the U.S. often carry a high diversity and heavy load of pathogens and parasites [63]. We found this to be the case in both years of our study. Both tracheal mites and Varroa mites were abundant, but not equally across all locations. Molecular markers for 5 viruses were common with relatively high copy numbers, while markers for 4 viruses were either not detected or not prevalent and usually were represented by low copy numbers. The recently (2011) discovered Lake Sinai virus [64] was at high prevalence (present in all sampled colonies in all locations) in 2015 (but not surveyed for in 2014). Other studies

have found similar high prevalence and incidence along with evidence suggesting that virulence can be high [65,66].

We were surprised to observe infestation of the parasitic phorid, *A. borealis* in 2015 at all sites ranging from 2.2-10.6% parasitism (average 5.5%). We did not detect it in 2014. In 2012, this parasite was detected in commercial colonies in South Dakota; San Francisco, California; and the Central Valley of California with parasitism levels at 12-38% [67]. It has been reported in the published literature in the U.S. since our study in 2015, but at low parasitism levels of 1-5% [68]. Whether this parasite is still common in commercial honey bee colonies in the U.S. is unknown. It has also been reported as a new parasite of the honey bee in Belgium [69] and Egypt [70]. Another new pathogen of fairly high prevalence and incidence (copy number) in our study was the trypanosome *L. passim*. This pathogen was described in the western honey bee in the U.S. in 2015 (71) and it has since been found to be detrimental to colony health [72]. With this rich diversity of pathogens and parasites, why was Varroa mite found to be the only significant causal factor in colony health? We speculate that because of the high level of co-occurrence of many of these pathogens in a colony and with the presence of the immune system compromising Varroa mite [73], pathogens become highly prevalent and abundant. Therefore, it is difficult to tease out a single causal pathogen agent and at a hierarchical level, high Varroa mite infestation in a colony represents a colony that has severe multiple pathologies of potentially different composition which ultimately can lead to a decline in colony health. The only constant or "Holy Grail" appears to be Varroa mite.

5. Conclusion

Health of migratory honey bee hives brought to wild blueberry for pollination was observed to vary over the two years that we conducted the study. Varroa mite infestation levels and pesticide residues in pollen (as measured by a Hazard Quotient) accounted for 57.8% of the variance in colony percent population growth rate during bloom. In general, pesticide residues other than miticides for the control of Varroa were common, but were not responsible for explaining a significant proportion of the variation in the percent change in sealed brood (P<0.498) when miticides were taken out of the Hazard Quotient). Tracheal mite and many of the pathogens were common in both years, but were also not significant causal factors of colony health. Therefore, it appears that Varroa mite is the main factor responsible for colony health of migratory hives brought into pollinate wild blueberry. This is because the trapped pollen Hazard Quotient we identified as a causal factor is most likely the result of Varroa mite control prior to and during pollination.

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Conflicts of Interest: The authors declare no competing interests.

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