

Article

Longitudinal Analysis of Honey Bee Colony Health as a Function of Pesticide Exposure

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Abstract: Sixty commercial honey bee colonies were monitored over the course of one year with the goal of assessing potential correlations between measured colony strength and environmental stressors, including exposures to pesticides and pathogens. We developed a new method for assessing colony health by determining the fractional change in population of the four colonies on each pallet between peak population on 1 June and the last population assessment prior to winter on 1 October. This fractional change in population was evaluated as a function of pesticide load per pallet for each of the 37 pesticide chemicals detected, grouping pallets by beekeeper. The analysis of individual chemicals showed that both imidacloprid and cyprodinil were negatively correlated with colony health, while tau-fluvalinate and dinotefuran (at very low concentrations) were positively correlated, possibly because of effects on *Varroa* control. Exposure to groups of chemicals was also evaluated. Normalization of each chemical concentration to the maximum observed for that chemical provided an equal weighting for each chemical, even though their relative occurrence in the environment and their effective toxicities might differ. A total of 24 chemical groups whose members share a structural commonality, a functional commonality, or suspected synergistic actions were considered, demonstrating negative correlations between colony health and exposures to neonicotinoids as a group and neonicotinoids in combination with (1) methoxyfenozide (2) organophosphates, and (3) diflubenzuron. Analysis of several groups of fungicides applied to almonds during pollination also showed negative correlations with colony health.

Keywords: honey bees; pollinators; pesticides; colony collapse; pollination



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1. Introduction

Since 2006, commercial beekeepers have experienced extensive colony losses, both during the overwintering period and, recently, during the summer as well [1]. Beekeepers are currently mitigating these colony losses at significant cost in resources and staff time [2], which strains the sustainability of commercial beekeeping operations in the U.S. The stresses experienced by honey bees affect not only managed bees and the beekeepers who rely on them for their livelihood but also wild pollinators, farmers that depend on pollinators, and ultimately our civilization that depends on a bountiful and diverse food supply.

Many potential causes of colony decline among the honey bees in North America, Europe, Asia, and the Middle East have been proposed [3–6]. These include exposure to agricultural chemicals, climate change, lack of diverse forage, hive management practices, and a proliferation of pests and pathogens. No single factor has been definitively identified as the primary cause of the recent increase in annual honey bee colony losses. Indeed, the interdependence of these factors cannot be dismissed. For example, certain pesticides have

been shown to down-regulate genes that control the function of the immune system [7], which is essential for protecting the honey bee from viruses transmitted by the *Varroa* mite. Determining the contribution of the different factors to the problem of colony losses is paramount to solving it and requires longitudinal studies to evaluate multiple colony stressors over time, as well as their potential interactions.

1.1. Relevant Prior Work

Previous work has demonstrated the necessity of longitudinal tracking of multiple parameters that may affect colony health. Van Englesdorp et al. conducted a comparison of multiple stressors (pesticides, pathogens, and parasites) on collapsed colonies vs. healthy colonies, but only at a single point in time [8]. Stoner and Eitzer evaluated pesticide residues in Connecticut colonies once per year over a two- to five-year period, documenting the presence of 60 different pesticides or metabolites in pollen [9]. More recently, Traynor et al. tracked the presence of pesticides in pollen over a 7-year period, with samples taken from a subset of colonies randomly surveyed for the National Honey Bee Disease Survey (NHBDS) to determine a baseline of pesticide exposure [10]. However, because the loads of pesticides and pathogens change over the course of a year, and their effects on colony health may not appear until weeks or even months after exposure, single-point analyses of a colony are insufficient for determining cause and effect.

Runckel, Flenniken, et al. tracked pathogen loads for a migratory bee operation, assessing their changes over time and correlations to colony health [11,12]. This study was the first of its kind and showed that pathogen types and loads in commercial honey bee colonies vary over time, even in visibly healthy colonies. The U.S. National Honey Bee Disease Survey conducted a longitudinal study of pathogens in both migratory and stationary honey bee colonies between 2009 and 2014, which established a baseline of honey bee diseases and their seasonality in the U.S. [13]. Further work by Faurot-Daniels et al. [14] also shows annual variation in pathogen populations. Measurement of pesticide residues and *Varroa* mite loads was not within the scope of these studies, and thus no conclusions could be drawn regarding potential for interactions with other stressors.

Using samples from the study presented here, Glenny et al. explored potential correlations of colony health as a function of the prevalence of seven common pathogens detected in the colonies, finding no statistically significant correlations between colony strength and pathogen prevalence for the primary pathogens known to infect honey bees [15]. Much work remains to be done to understand the interactions between honey bee pathogens and other colony stressors [16].

Pesticides, including insecticides, herbicides, and fungicides, have garnered particular attention as potential stressors because of their known toxicity and widespread use in crop production. Multiple studies show that honey bee exposures to pesticides are common and that exposures change depending on the season of application, the availability of contaminated pollens and nectars, and the season in which they are consumed [17,18].

The neonicotinoid insecticides have received the most intensive study because of their high acute toxicity to honey bees, persistence in the environment, and their high water solubility, which facilitates systemic uptake and distribution into pollen and nectar, thereby increasing exposure potential for pollinators. Sublethal effects of neonicotinoids have been observed at environmentally relevant concentrations and include impairment of immune function [7,19], reduced queen lifespan and fertility [20,21], decreased sperm viability in drones [22,23] interference with foraging ability [24–26] and navigation [27], and reduced brood survival [28]. Recent work indicates that sublethal exposure can affect reproduction in native bee species over months to years [29,30].

Certain fungicides, while not acutely toxic to bees, may also impair colony health [31,32]. Mussen documented beekeeper reports of time-delayed brood kills after fungicide applications to almonds [33]. McArt et al. found a landscape-scale correlation between use of fungicides and increased *Nosema* prevalence in bumblebees [34], a result consistent with the observation of Pettis et al. of increased probability of *Nosema* infection in honey bees

consuming fungicide-contaminated pollen [35]. Yoder et al. indicate that fungicide contaminants reduce the diversity and number of beneficial fungi essential for the conversion of pollen into bee bread within the hive, with potential to impact the nutritional status of the colony [36].

Fungicides have been observed to interfere with the production of cytochrome P450 enzymes responsible for detoxification of both naturally occurring and synthetic chemicals in the honey bees' environment [37–39]. Mao et al. found that azole fungicides bind to a cytochrome P450 (CYP) enzyme (CYP9Q1) responsible for metabolism of quercetin and other naturally occurring components of pollen, reducing the ability of the bee to process these components of its diet [40]. The result was impaired mitochondrial regeneration, resulting in production of less ATP as energy for flight muscles. Traynor et al. found that the presence of fungicides that inhibit sterol biosynthesis (azoles) and those with multi-site contact activity were correlated with queen failures [5]. Ricke et al. found that fungicides used during almond bloom reduced queen longevity [41].

Insect growth regulators impair honey bee colony growth and longevity, with effects ranging from mortality of larvae and pupae to physical abnormalities in exposed adult bees [42], as well as reduced hypopharyngeal gland development [43], inhibition of vitellogenin synthesis [44], and reduced forager survival [45].

Reductions in CYP enzyme function can also enhance the toxicity of environmental chemicals by slowing metabolic detoxification pathways. Indeed, the addition of a “synergist” that disables insect detoxification systems has long been used as a resistance management tool, with the role of CYP enzymes in this process elucidated long after the effect was known [46]. Johnson et al. measured up to 100-fold increases in the toxicity of insecticides in the presence of azole fungicides [47]. Similar results have been noted by Han et al. for Asian honey bees [48]. Sgolastra et al. noted a synergistic toxic effect between clothianidin and propiconazole in both honey bees and native bees [49]. Synergies have been noted by Tosi et al. between the butenolide neonicotinoid insecticide flupyradifurone and propiconazole as well [50]. Indeed, a substantial number of fungicide patents document synergistic enhancement of insecticidal toxicity as a characteristic [51].

1.2. Study Background and Goals

In this paper, we describe a study in which we conducted longitudinal monitoring of 20 colonies in each of three commercial beekeeping operations (60 total colonies) from January 2014 to January 2015. Commercial beekeepers offer a statistically large population of honey bees that are exposed to a variety of environments during each year, enabling temporal measurements of colony stressors in the hive along with colony health to search for correlations that may illuminate fundamental causes of colony failures. The goals of the study were to assess the dependence of colony health and survival on the occurrence of specific pests, pathogens, and pesticides or combinations of pesticides found in the 60 colonies over time. The results of the dependence of colony health on a range of pathogens have already been published, with no statistically significant correlations found between colony health and any specific pathogen [15]. This current paper focuses on assessment of colony health as a function of pesticide concentrations and mite loads.

In the spring of 2014, significant colony losses were experienced by many commercial beekeepers after pollinating almonds, with numerous reports of dead or malformed brood during and immediately after almond bloom [52]. There was speculation that application of a fungicide/insect growth regulator (IGR) tank mix was responsible for the spring losses. Wade et al. conducted a follow-up *in vitro* bioassay to test this hypothesis, evaluating the effects on larval survival of two insect growth regulators and an insecticide (diflubenzuron, methoxyfenozide, and chlorantraniliprole) and four fungicides (propiconazole, iprodione, and a combination of boscalid and pyraclostrobin), both singly and in combination [53]. The results demonstrated increased larval mortality after exposure to the insect growth regulator diflubenzuron alone and to combinations of propiconazole or iprodione with chlorantraniliprole. Neither methoxyfenozide nor any methoxyfenozide-fungicide com-

bination increased mortality. Fisher et al. studied the effects of combined exposure to methoxyfenozide, pyriproxyfen, and bifenazate in a laboratory setting, finding adverse effects on foragers [45]. These results are consistent with the pattern of 2014 losses, but the studies did not evaluate the full range of pesticides used on almonds. The results presented here indicate that other fungicides and combinations of fungicides and other pesticides may be equally or more problematic for colony health than those tested previously.

2. Materials and Methods

Sixty honey bee colonies from three different commercial migratory beekeeping operations were included in this study and monitored for one year, from January 2014 to January 2015. Colony strength, chemical concentrations in the pollen, pathogen loads, and *Varroa* mite counts in the hives were measured at various times throughout the year. Beekeeper-specific details on colony management are provided in Appendix A.

2.1. Colony Selection and Study Design

Three commercial beekeeping operations were included in this study, identified as “JA”, “SE”, and “DC”, associated with co-authors of this paper. Each beekeeper contributed twenty honey bee colonies for the study, with four colonies on each of five pallets, thus incorporating 15 pallets supporting 60 colonies. Pallets were coded by beekeeper, as JA-1, JA-2, etc. At the start of the study, all colonies were queen-right and had successfully survived the 2013–2014 overwintering period. Colonies selected for the study contained on average 9 ± 2.6 frames of bees, based on the conventional frame-count method used in contracts for commercial pollination to assess hive strength—the number of frames within a hive having a minimum number of bees [54,55].

2.2. Sampling Hive Materials for Pesticides

Pollen as beebread was sampled for pesticide analysis four times during the year: (1) Before almond pollination (February 2014); (2) Immediately after almond pollination (March 2014); (3) Between April and June 2014; and (4) In early October 2014. For each hive, 3-inch core samples of a comb near the brood nest containing wax, pollen, and honey were extracted for pesticide residue analysis. Composite pollen samples were prepared for each pallet from the ensemble of individual hive cores by mixing equal masses of pollen from each of the available hives to create a single homogeneous 3 g sample for residue analysis. Sampling details, colony locations, and colony characteristics and management are described in Appendix A.

2.3. Laboratory Pesticide Analysis

Hive matrix samples (pollen/bee bread, wax, honey) were analyzed by the USDA lab in Gastonia, NC using the QuEChERS-NSL (National Standard Lab) extraction procedure for a multi-residue screen for approximately 180 different chemicals, including pesticides and common metabolites [56]. Analytes, their detection limits (LODs), current US EPA registration status, use type, and chemical classifications can be found in the Supplementary Material, Table S1. Over the course of the project, spiked samples were also prepared and sent to the USDA lab to validate laboratory accuracy. Standards of azoxystrobin, chlorpyrifos, coumaphos, λ -cyhalothrin, cyprodinil, fluvalinate, imidacloprid, methoxyfenozide, pendimethalin, and thiamethoxam at 100 $\mu\text{g/mL}$ were purchased from AccuStandard and diluted to 20 $\mu\text{g/mL}$ stock solutions. Three pollen samples of known residue composition were spiked by adding the appropriate amount of stock solution for several different pesticides typically found in the samples to produce concentrations in the range of the analytical method. All spike results were within $\pm 30\%$ of the spiked value, indicating that the analyses were reliable within the limitations of the method. Recoveries of the spiked amounts averaged 110% (SD = 27%).

A subset of the samples (early summer and late summer samples) was submitted to the Harvard Center for Environmental Health laboratory for a higher-sensitivity analysis of neonicotinoids in pollen, with limits of detection (LOD) 2–5-fold lower than those of the USDA laboratory. A modified QuEChERS method was used for the extraction, as described in Chen et al. [57]. One pollen sample spiked with known amounts of neonicotinoid pesticides was sent to the Harvard lab. Recoveries ranged from 74% to 79% of the spiked amount, averaging 76% (SD = 4%). Project samples found to have imidacloprid and clothianidin concentrations between 1 ppb and 7 ppb by the Harvard lab were systematically identified as <LOD by the USDA lab, even though the USDA LODs were listed as 1 ppb, indicating systematic inaccuracies in the USDA methods at lower concentrations.

2.4. Measuring Changes in Colony Strength over Time

Colony strength was measured at 7 to 10 different times, depending on beekeeper, during the period from January 2014 to January 2015. The bee population was measured by using “frame counts” as the metric of colony strength, as described and listed in Appendix B within Tables A1–A3.

A total of 35 of the 60 colonies were split in the spring of 2014. For colonies that were split, the old queen was left with the original study hive, so there was no break in the brood cycle. The split was moved away from the study hive pallets and was no longer considered part of the study, except as described here. Frame counts from splits were accounted for by calculating a split factor (SF), defined as the number of frames remaining in the original hive after the split divided by the total number of frames in the original hive, e.g., for an 18-frame colony from which a 4-frame split was made, $SF = 14/18 = 0.78$. Corrected frame counts were calculated by dividing the measured frame counts by SF for all frame count measurements made after the split. This correction assumes that the population of the split changes at the same rate as the original colony. Many of the SE hives were split twice, necessitating the use of a second split factor, SF_2 , defined as the number of frames remaining in the split hive after the second split divided by the total number of frames in the original hive. The corrected frame count was calculated as the measured frame count divided by the product of SF and SF_2 .

In order to match results with composite pesticide samples across hives on a pallet, frame counts for all four hives residing on each pallet were summed to arrive at a pallet population. Summing frame counts over a pallet improves the statistical integrity of the measured bee population, reducing inter-colony variability due to queen performance and solving the mathematical problem of zero frame counts for individual colonies that died during the study.

The “bee population” (frame count) for each pallet was plotted as a function of time. A spline interpolation was computed between the (approximately) eight points of pallet population measurement to estimate the population in between actual measurements to produce a continuous curve of frame counts vs. time during the year.

Using these population curves, a metric of population change was computed: the percent change in the honey bee population between 1 June and 1 October. This percent change represents the fractional decline in the bee population between the early summer peak population and the fall season when a reduced population of a colony is preparing for winter. This metric of population change was defined as the total pallet frame count on 1 October minus the total pallet frame count on 1 June, divided by the pallet frame count on 1 June, i.e., the fractional change in the number of frames stated as a percentage.

2.5. Construction of a Metric of Chemical Exposure

A straightforward metric of exposure to each of the 37 chemicals was developed by summing the four measurements of concentration in the pollen from each pallet. The table of these 37 exposure metrics for each pallet is given in Appendix C, as Table A4.

In order to search for correlations between the fractional change in bee population and the combined group concentrations of several chemicals with similar modes of action, a simple metric based on concentrations normalized to the maximum observed concentration was constructed. Each pesticide concentration was divided by the maximum observed concentration ever measured in this study for that chemical. Normalization gives each chemical equal weight, even though their relative occurrence in the environment and their effective toxicities might differ. For each chemical group and pallet, the max-normalized concentrations of each chemical were summed over the entire study period to obtain an estimate of exposure. A total of 24 chemical groups whose members share a structural commonality, a functional commonality, or suspected synergistic actions were considered (see Sections 3 and 4).

2.6. Assessment of Potential Correlations

Potential correlations of colony health to chemical exposure were assessed by plotting the fractional change in the bee population vs. the sum of concentrations (in ppb) for individual chemicals and the sums of the max-normalized concentrations (unitless, see above) for groups of chemicals. There is a clear grouping of data points by beekeeper, likely reflecting beekeeper-specific locations/exposures and management practices. Thus, we averaged the fractional change in bee population and the sum of the concentrations for each beekeeper and plotted these averages. Thus, each plot contains three points, with each point representing a beekeeper. The error bars represent the uncertainty in the mean of the two measured quantities from the five pallets. An unweighted linear least-squares fit was computed to provide a trendline for visual inspection. The limited number of beekeepers precluded a formal statistical assessment of significance.

2.7. Measurement of Varroa and Pathogen Prevalence

Varroa mites per 100 bees were counted in each hive 5–6 times during the year, depending on beekeeper, using either the ethanol wash [58] or sugar roll [59] method. Ultimately, it was not possible to use these data in statistical analysis due to the variability in *Varroa* management timing and methods used by the different beekeepers.

Adult hive bees were sampled five times during the year from selected colonies to assess the occurrence of pathogens, specifically. A single frame from the center of the brood nest covered with bees was selected and shaken into a bin. One-third of a cup of bees (approximately 150 bees) were placed in a pre-labeled quart-sized Ziploc® bag, immediately packed in dry ice for transport, then placed in long-term storage at -20°C . The analytical methods used to assess pathogen presence and the results of this analysis are described in reference [15].

3. Results

3.1. Colony Population Changes with Time

Figure 1 shows plots of the bee population over time during the study, as experienced by the 15 pallets from all three beekeepers. The points show the measured bee populations for a specific date, and the dashed line represents the spline interpolation between the points.

The percentage change in bee population between 1 June and 1 October for the 15 pallets is shown in Figure 1. The populations exhibit the well-known seasonal pattern of an increase from February to May, a plateau during early summer, and a decline in late summer and early autumn. We use this percentage population change for each of the 15 pallets for all upcoming analyses as a simple and transparent metric related to the change in the health of the bees from early summer to fall. The percentage changes in bee populations are negative except for two, DC-2 and DC-5, which nonetheless suffered their declines a few weeks after 1 October. On average, the percent population change for the 15 pallets is -43% and the median is -54% . A total of 28 of the 60 colonies (47%) died before the end of the study, seven from the DC operation, eleven from the JA operation, and

ten from the SE operation. The full data set of colony population, over time, is available in Appendix B (as previously noted).

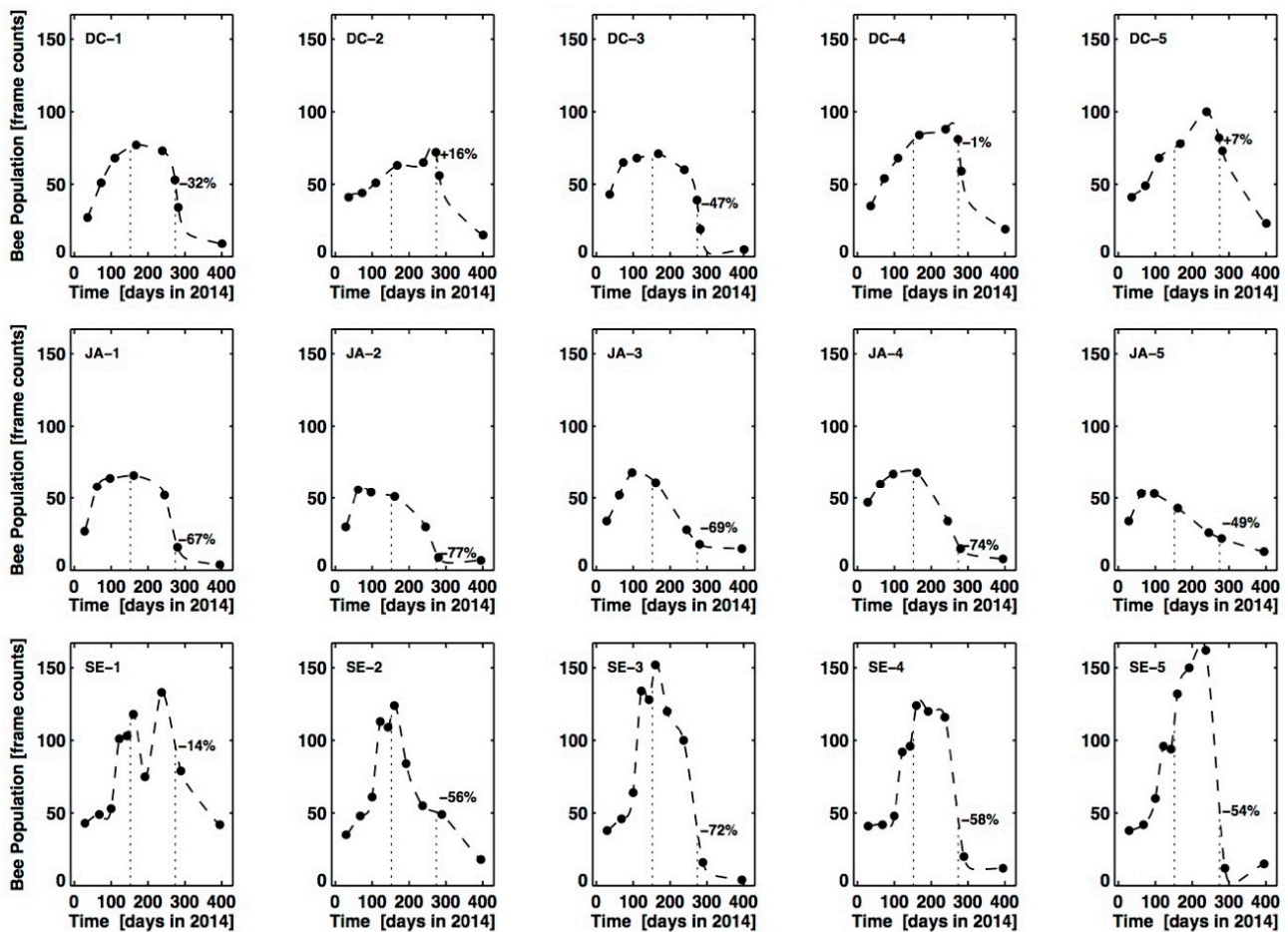


Figure 1. Bee population (in counts of healthy frames) vs. time during 2014 for all 15 hives in the study. The points represent the measured bee population in units of frame counts on each pallet. The dashed line shows a spline interpolation. Two vertical dotted lines mark the dates 1 June and 1 October, during which the percentage change in the bee population (in frame counts) is noted at the right.

3.2. Pesticide Concentrations

Analytical results revealed the presence of 37 different pesticide chemicals in hive pollen over the course of the study that exhibited at least one measurement significantly above the LOD. Ten of these chemicals had either three or fewer significant detections across all samples, namely captan, cypermethrin, flubendiamide, myclobutanil, pyrimethanil, pyriproxyfen, spiroticlofen, tebufenozide, tetrahydrophthalimide, trifloxystrobin, and tri-fluralin. Thirteen additional pesticides or degradation products were detected in trace quantities at or near the LOD, including aldrin, bifenthrin, chlorferon, DDE, endosulfan II (beta), fenpropathrin, fenpyroximate, metalaxyl, norflurazon, phorate, quinoxifen, sethoxydim, and tebuconazole. The data were insufficient to conduct a statistical analysis for these 23 chemicals and they are not included in the analyses described below in Figures 2–4. A comprehensive table of results by pallet is given in Appendix C, Table A4.

Of the 37 pesticides detected, 33 are still in current use, one (flubendiamide) was canceled by US EPA in 2016 for excessive risk to aquatic ecosystems, and the remaining three chemicals (coumaphos-oxon, THPI, and 2,4-DMPF) are degradation products of registered pesticides (see Supplementary Material, Table S1).

At the time of the first measurement in February 2014, before the hives went to almond orchards, the pollen analyzed was largely free of pesticide residues (see Table 1). The miticide thymol and the amitraz metabolite 2,4-DMPF were most frequently detected, along with the herbicides pendimethalin and oxyfluorfen at modest levels.

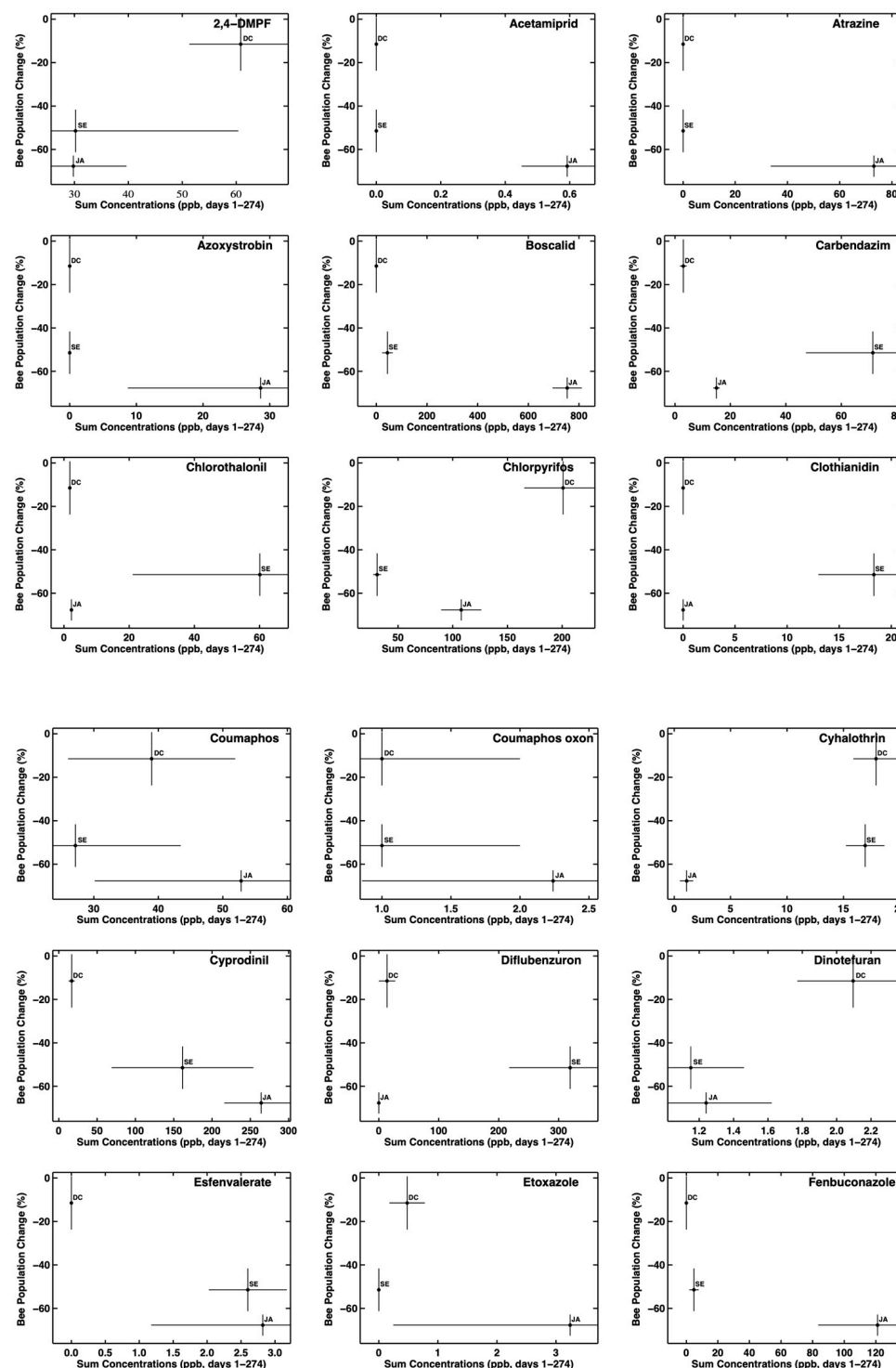


Figure 2. Cont.

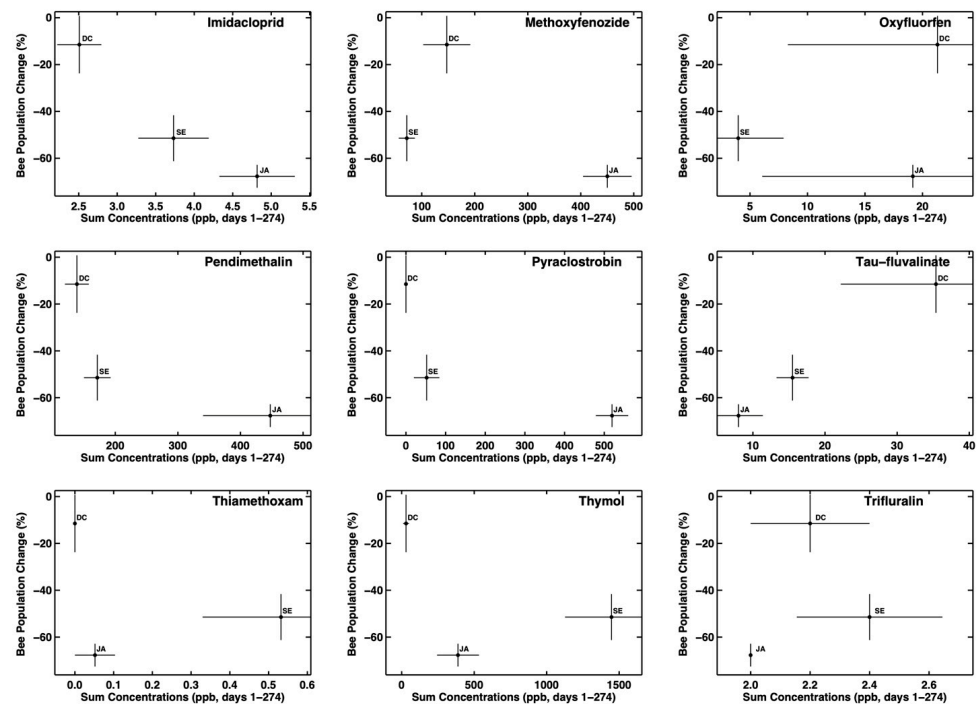


Figure 2. Bee population change from 1 June to 1 October vs. the sum of chemical concentration measurements. Each panel represents a specific chemical. Each point represents one beekeeper and the average concentration of the chemical over five pallets containing four hives. The error bars show the uncertainty in the mean.

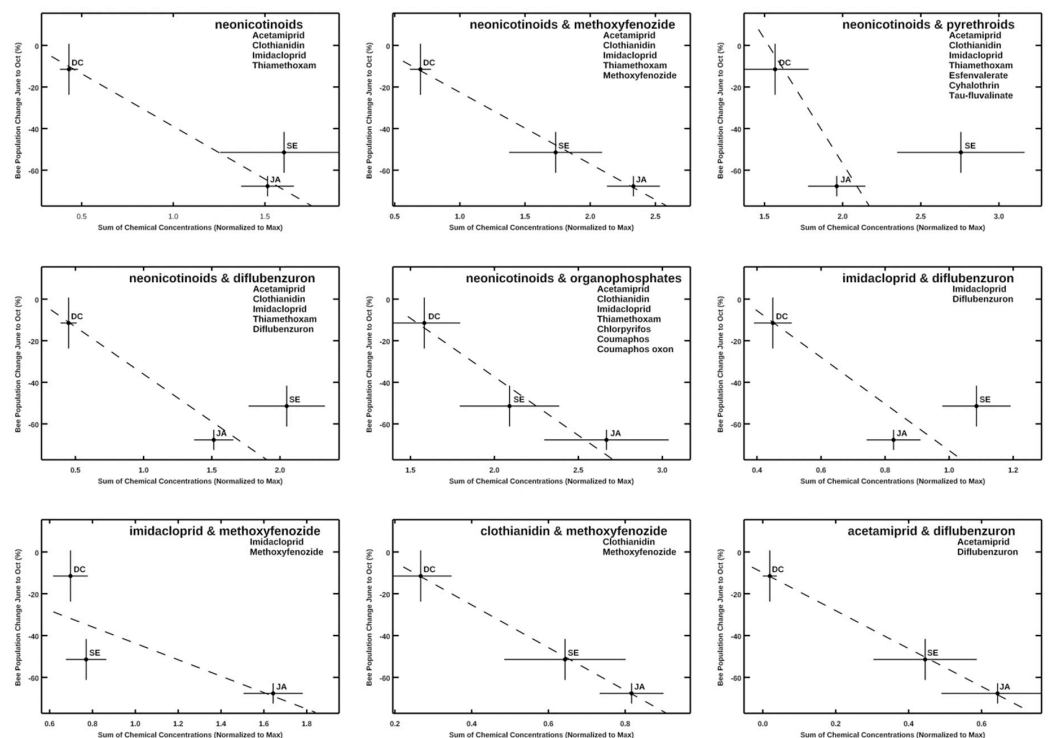


Figure 3. Neonicotinoid groups. The plots show bee population change vs. concentrations of combinations of neonicotinoids and other chemicals. Each plot shows the percent change in the frame count from 1 June to 1 October vs. the sum of chemical concentration measurements (max-normalized, see text) for the group of chemicals listed at upper right. Each of the three points represents one beekeeper. The dashed line is the best straight-line fit to the data including errors in both quantities.

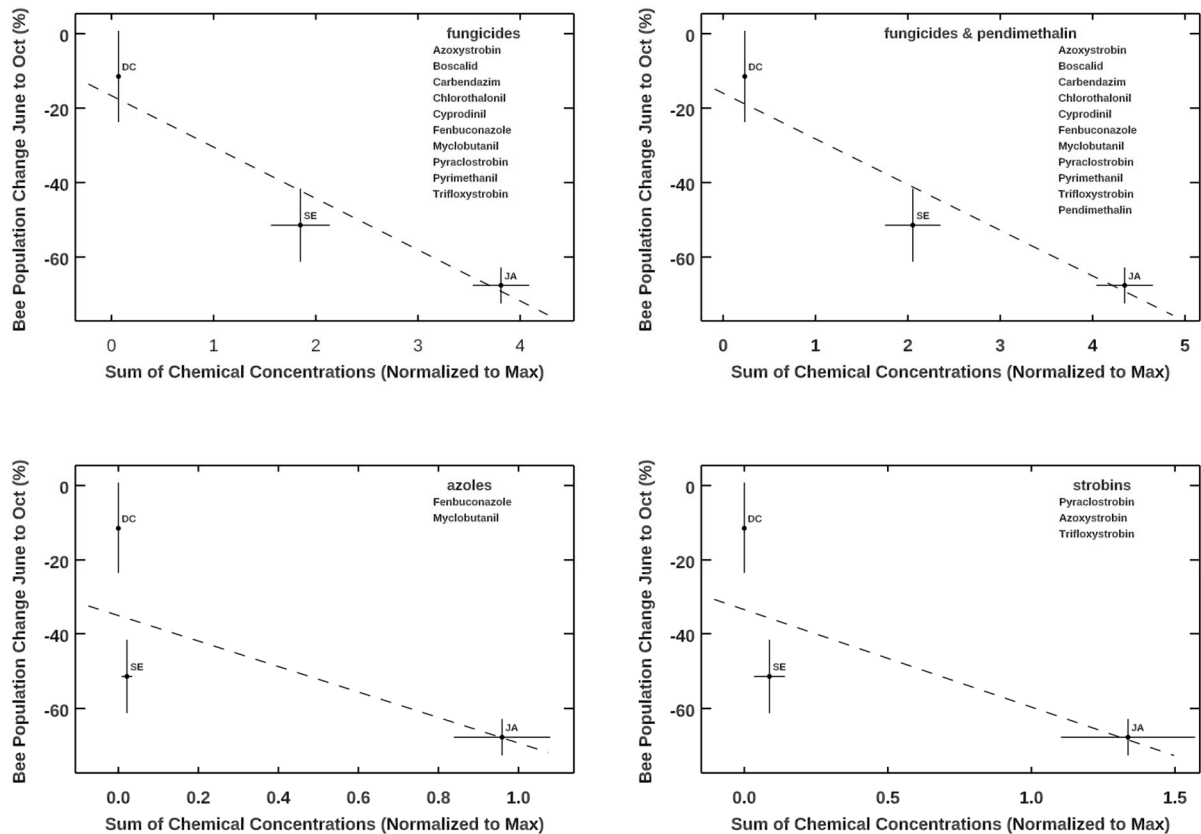


Figure 4. Fungicide groups. The four plots show bee population change vs. concentrations of fungicide groups, as with Figure 3 but with fungicides. The sum of concentrations of all four groups of fungicides correlates with the fractional change in bee population from June to October. Thus the fractional decline in honey bee population is negatively correlated with increasing fungicide concentration.

The measurements made after the colonies were removed from the almond orchards revealed 17 different chemicals in the pollen samples from the 15 pallets, including insecticides, fungicides, and insect growth regulators.

Although pallets from each beekeeper were placed in different almond orchards in February (with the exception that SE-1 and SE-4 were placed with the JA pallets), the mix of pesticides found in the post-almond pollination samples from each beekeeping operation was similar, dominated by pesticides applied during almond bloom. Most of the pesticides detected were permitted by law to be applied to almonds during bloom; others are likely a result of bees foraging in nearby fields. Once the beekeepers left California for their different summer locations, the types and amounts of pesticides detected varied substantially across operations. Some pesticides were found only in the hives of a single beekeeper.

Data collection for one of the beekeepers (JA) allowed the assessment of pesticide exposure over several months. The JA hives were moved to cherries immediately after pollinating almonds, and samples were taken both after almonds (in March) and after cherries (in April). An additional set of samples was taken in June, when all JA hives appeared to be in crisis (dead bees in front of the hives and queen failures). These samples contained several of the same pesticide residues detected both after almond pollination and after cherry pollination (methoxyfenozide, boscalid, pyraclostrobin, pendimethalin, chlorpyrifos, and thymol), indicating continuous exposure to these chemicals over the course of several months. The highest absolute concentrations over time were measured for boscalid, pyraclostrobin, methoxyfenozide, and pendimethalin.

Table 1. Pesticide detection before and after almond pollination.

	Before Almond Pollination				After Almond Pollination		
	LOD (ppb)	Avg Conc. (ppb)	Conc. Range (ppb)	Detection Frequency (%) ^a	Avg Conc. (ppb)	Conc. Range (ppb)	Detection Frequency (%) ^a
Fungicides							
Boscalid	4	ND	ND	0%	283	19–821	67%
Carbendazim	5	ND	ND	0%	33	5–124	87%
Chlorothalonil	1	1	1	73%	22	1–195	93%
Cyprodinil	1	ND	ND	0%	129	10–476	100%
Etoazole	1	1	1	13%	8	1–15	13%
Fenbuconazole	10	ND	ND	0%	76	10–214	47%
Pyraclostrobin	2	ND	ND	0%	243	15–554	67%
Pyrimethanil	3	ND	ND	0%	4	3–5	20%
Tetrahydrophthalimide ^b	50	ND	ND	0%	50	50	7%
Herbicides							
Oxyfluorfen	1	19	4–37	20%	42	20–65	13%
Pendimethalin	6	50	6–81	27%	153	85–233	100%
Trifluralin	1	1	1	7%	1	1	100%
Insecticides							
Acetamiprid	0.05	ND	ND		1	ND–0.92	33%
Chlorpyrifos	1	3	1–8	33%	48	12–123	100%
Cyhalothrin, lambda	1	1	1	13%	4	1–6	53%
Cypermethrin	4	ND	ND	0%	4	4	7%
Dinotefuran	1.00	ND	ND	0%	1	1.00–1.97	60%
Esfenvalerate	2	7	7	7%	2	2	20%
Imidacloprid	1.00	ND	ND	0%	4	1.67–5.83	100%
Spirodiclofen	1	ND	ND	0%	5	5	13%
Insect Growth Regulators							
Diflubenzuron	10	ND	ND	0%	277	69–717	40%
Flubendiamide	25	ND	ND	0%	558	182–863	20%
Methoxyfenozide	2	ND	ND	0%	133	41–350	100%
Pyriproxyfen	1	ND	ND	0%	6	6–7	13%
Tebufenozide	5	ND	ND	0%	30	30	7%
Miticides and their Degradates							
2,4-DMPF ^c	4	41	20–65	53%	24	15–34	20%
Coumaphos	1	5	3–14	40%	18	3–92	93%
Coumaphos oxon	5	5	5	7%	ND	ND	0%
Tau-fluvalinate	1	16	7–36	27%	12	4–43	87%
Thymol	50	175	50–709	67%	461	50–2310	80%

ND = Not detected. ^a Detection frequency reported as a percentage of 15 pallets tested. ^b Chlorothalonil degradation product. ^c Amitraz degradation product.

Analysis of the individual hive samples allowed for an assessment of the variability of pesticide measurements in pollen across hives in the same location and in different locations. The data indicate that while concentrations may vary by a factor of up to 11 across individual hives, pallet concentrations only vary by up to a factor of 3.6 (see Appendix C).

3.3. Reproductive Failures

Observations of reproductive failures were noted throughout the study, including queen supersedure, poor brood pattern or no brood, drone-laying queens, or laying worker bees. All of these observations are indicative of colony exposure to stressors that impair the queen’s ability to reproduce or the larval ability to grow and mature into adult bees. Over

the course of the study, 42 colonies exhibited symptoms of reproductive failures at some point during the study, and 18 did not. Of the colonies that did not experience reproductive failures, 82% survived to the end of the study. In contrast, only 64% of the 42 colonies experiencing reproductive failures survived to the end of the study.

3.4. Correlation of Fractional Change in Bee Population with Individual Chemicals

Figure 2 contains 27 plots, each corresponding to an individual chemical, showing the percentage change in the bee population from 1 June to 1 October vs. the measured metric of chemical exposure, as specified above. Each plot contains three points, each point representing a beekeeper. The error bars are the uncertainty in the mean of the quantities from the five pallets for each beekeeper.

Figure 2 indicates that several individual chemicals exhibit a correlation between the specific chemical concentration in the hives and the fractional change in bee population. The plots for imidacloprid and cyprodinil show the strongest negative correlations between concentration and fractional declines in bee populations. Tau-fluvalinate and dinotefuran exhibit a positive correlation, with a lesser decline in bee population with a greater exposure to the chemical. Robust *p*-values cannot be computed from three data points, but the fit to a linear least-squares trendline is tight.

3.5. Correlation of Fractional Change in Bee Population with Groups of Chemicals

In Figures 3 and 4 we plot the average percent change in population from 1 June to 1 October vs. the average sum of max-normalized concentrations of chemicals for the five pallets of each of three beekeepers, with the included substances listed at the upper right of each panel.

Figure 3 shows combination plots for different neonicotinoids, as well as neonicotinoids combined with other chemicals to search for potential synergies. All plots exhibit a downward trend, indicating the fractional decline in the bee population was greater with increasing exposure to neonicotinoids or neonicotinoids plus other chemicals.

The dashed lines in Figure 3 show the best-fit straight line that includes the uncertainties in both dimensions, i.e., in the sum of the max-normalized concentrations of the neonicotinoids and in the percent bee population change from the five pallets of each beekeeper. The uncertainty in the mean of the five pallets captures the fluctuations for a given beekeeper, caused by colonies bringing in or consuming different amounts of pesticides and/or sampling inhomogeneity.

The observed downward correlation between the change in bee population and the increasing amount of neonicotinoids appears significant, as the population decline exceeds the size of the error bars and the straight line fit goes through the error bars. A robust *p*-value is not possible due to the paucity of beekeepers, i.e., only three.

Figure 3 also contains a similar diagnostic plot for eight other groups of chemicals that include neonicotinoids, five of which exhibit a downward trend. The five groups are neonicotinoids plus methoxyfenozide, neonicotinoids plus diflubenzuron, imidacloprid plus methoxyfenozide, clothianidin plus methoxyfenozide, and acetamiprid plus diflubenzuron. The persistent downward trends suggests that neonicotinoids in combination with the other chemicals are associated with greater end-of-season bee population decline (but this is not necessarily causal; see Discussion). We note that dinotefuran is not included with the set of neonicotinoids in this analysis because of its previously noted positive correlation with population change that may be due to its deleterious effects on *Varroa* at concentrations that appear to have minimal effects on honeybees.

Figure 4 shows plots for chemical groups containing fungicides and one herbicide. The first panel of Figure 4 at upper left shows the percent bee population change vs. the sum of max-normalized concentrations of ten fungicides, with the other three plots providing data on groups of strobilurin and azole fungicides, and fungicides plus the herbicide pendimethalin. The persistently greater fractional declines in bee population with increased fungicide concentrations in all four combinations supports the authenticity of the correlation. Again,

the presence of only three points and their error bars are insufficient to perform a robust statistical test of the significance of the correlation.

4. Discussion

The goals of the study were to assess the dependence of colony health on the occurrence of specific pests, pathogens, and pesticides or combinations of pesticides found in the 60 colonies tracked over one year. We were not able to determine dependence of colony health on *Varroa* populations because of the substantial differences in methods of mite management used by the different beekeepers. The results of the dependence of colony health on a range of pathogens have already been published [15], with no statistically significant correlations found between colony health and any specific pathogen. Below, we discuss the implications of the pesticide results obtained in this study. It is worth noting that because most of the commercial bee population in the U.S. is transported to California for almond pollination, and because similar pesticides are used across different almond orchards, our results apply more broadly to the population of honey bees used to pollinate almonds.

4.1. Exposures over Time

As pesticide concentrations within a hive change substantially over time, frequent measurements are required to determine exposures as hive resources are consumed or stored. There can be a time lag between when chemicals enter the hive and when they are consumed and/or exhibit observable effects. For example, if exposure affects the queen and her egg-laying ability, the 21-day brood maturation period must pass before colony population changes significantly. During times when uncontaminated pollen is abundant (such as for the SE colonies in Angels Camp), previously stored pesticide-laden pollen may not be consumed immediately, further delaying any observable effects. These factors complicate determination of a direct cause and effect from pesticide-contaminated pollen.

The duration of exposure to a chemical also affects toxicity. A short-term high-dose exposure may be tolerable by an organism, while a longer-term exposure of a lower dose may be toxic [60,61]. The JA colonies that pollinated almonds and cherries contained pollen with substantial levels (tens to hundreds of ppb) of methoxyfenozide, boscalid, pyraclostrobin, and pendimethalin over several months. Assuming the bees consumed the available pollen in the hive, the “dose” of these chemicals received by the colony lasted not a single day or week but instead lasted at least several months between March 3 and 1 June. For worker bees in the spring, with an average life span of 6–8 weeks, the presence of these chemicals in the hive for three months represents a lifetime of exposure for several generations of honey bees and a substantial fraction of a queen bee life span. The fact that these four pesticides (and others as well) were present in the colonies simultaneously over the same time period also provided ample opportunity for any synergistic toxic effects to occur. Our simple sum of concentrations during the year has the advantage of capturing some measure, however sub-optimal, of the two temporal and dosage regimes of exposure: brief, high-dose exposures causing acute effects and chronic exposures to sublethal doses associated with a variety of adverse effects which impair colony health and reproduction.

4.2. Variability in Pesticide Measurements

There are several sources of variability in the pesticide measurements from the different study hives, including the inhomogeneity from sampling only a single core sample from each hive, the inhomogeneity of the pollens inside the cells selected for analysis, the different foraging patterns of the colonies, the date the contaminated pollen was brought into the hive, the date on which the pollen was consumed, and the different consumption rates of the pollen within the colony. None of these variables can be controlled, hence our decision to average the observed pesticide residue concentrations over the four hives on a pallet, reducing variability from a factor of 11 across the 20 hives placed in the same location to a factor of 3.6 across the five pallets (see Appendix C).

4.3. Correlation of Fractional Change in Bee Population with Pesticide Exposure

We constructed simple metrics of both the fractional change in the bee population from 1 June to 1 October and the year-long exposure to various pesticide active ingredients, allowing us to perform transparent searches for any correlation between colony health and exposure to pesticides. The diagnostic plots of the fractional change in the bee population versus exposure to individual chemicals (Figure 2) suggested negative correlations of colony health with exposure to cyprodinil and imidacloprid. Only two chemicals showed a possible positive correlation, tau-fluvalinate and dinotefuran. Tau-fluvalinate is used as a miticide, which likely explains this result, as it may have limited the population of *Varroa*. The same may be true of the low levels of dinotefuran found (1–2 ppb). None of these results can be considered statistically significant due to the limited number of beekeepers involved in the study (only three). Future longitudinal studies including more beekeepers at different locations with four to eight hives per beekeeper would enhance the statistical power of these types of studies.

The consideration of groups of chemicals that share common molecular structure, mode of action, or known biological synergies allowed for a more statistically powerful search for correlations, as shown in Figures 3 and 4. The inclusion of many chemicals within a group helped both illuminate any additive or synergistic effects of multiple sub-lethal doses and provided a sum of chemical concentrations having less fractional uncertainty than did any one chemical alone. The results for groups of neonicotinoids, fungicides, and insect growth regulators (Figures 3 and 4) revealed certain groups of chemicals that persistently correlated with a greater fractional decline in the bee population between the peak population in early June and early October. Specifically, the negative correlations observed for combinations of neonicotinoids and methoxyfenozide and neonicotinoids and diflubenzuron are consistent with laboratory experiments of synergies between these chemicals, e.g., [37,62]. The calculation of a statistically robust *p*-value was not possible due to the limited number of beekeepers, three, in the study.

5. Conclusions

5.1. Pesticide Exposures Negatively Affect Honey Bee Colony Health

This study provided extensive data on pesticide concentrations in commercial honey bee colonies over the course of a year, as beekeepers conducted their normal pollination and honey-making activities. These data provide evidence of pesticide effects on honey bees at field-realistic doses and with typical commercial management practices. Our findings indicate that the fractional decline in honey bee populations from peak population in early June to a low in early October was systematically greater with increasing amounts of certain groups of chemicals examined. Groups with visible correlations include neonicotinoids as a group, neonicotinoids with methoxyfenozide, neonicotinoids with organophosphates, and fungicides as a group. While we cannot calculate a precise *p* value for these effects, the predominant negative change in fractional decline of the bee population with many of the chemical groups indicates the correlation is not governed by random chance. If there were no correlation, one would expect as many positive as negative correlations. Such was not the case. The fractional change in honey bee populations between 1 June and 1 October was systematically greater with increasing amounts of the groups of chemicals examined. The indications of correlations observed with fungicides and insect growth regulators merit further scrutiny of the sublethal effects of these pesticides.

5.2. Improving Methods for Assessment of Mixtures of Pesticides

Our methods for combining the concentrations of multiple chemicals brought value to this study in multiple ways. Assessing combinations provides a preliminary estimate of potential additive or synergistic effects of different pesticides. Bees may survive a sub-lethal dose of one chemical, but they may not survive the additive effects of sub-lethal doses of several chemicals. In addition, pairs of chemicals may act synergistically to impact the health of bees more than either chemical alone. Such synergies often involve

disabling of the cytochrome P450 detoxification mechanisms and have been found in other studies [46,47,49,51]. Indeed, many of these synergistic combinations of pesticides have been patented specifically for these synergies [51]. Combining the concentrations of several chemicals reduces the fractional noise of the sum of measured concentrations compared to the fractional noise of an individual chemical, thereby improving the detectability and statistical integrity of any correlations.

This study faced several challenges. One was constructing a useful metric or “formula” to compute a sum of concentrations of multiple chemicals. It was impossible to know what weight (i.e., coefficient) to assign to the concentrations of each chemical within a group when “adding” them together. Because only acute toxicity (LD_{50}) data are available for most chemicals, we also did not know the functional dependence of each concentration on bee health exclusive of morbidity, with nonlinearities being likely. We also did not know the range of sublethal effects of each chemical within a group, nor did we have comprehensive knowledge about synergies between different chemicals.

Still, by simply adding together the max-normalized concentrations of different chemicals, any correlation of this metric of chemical exposure with the fractional change in the bee population provides a clue about causal dependencies or stressors associated with those chemicals. While other factors besides chemical exposure could be the reason for population declines, the observed correlations are consistent with extensive laboratory work demonstrating that bees exposed to certain individual chemicals experience adverse effects on their health [5,6,17,19,47,49]. The correlations found in this longitudinal field study suggest that the deleterious health effects of these chemicals found in the lab do indeed occur in the field.

5.3. Protection of the Honey Bee Population

In order to protect honey bees from adverse effects due to pesticide exposure, a reduction in pesticide use on bee-attractive crops during pollination season would need to occur. In 2018, the Almond Board of California published a “Best Practices” guide for almond growers that describes potential problems related to synergistic effects from applying tank mixtures of fungicides and/or insect growth regulators (IGRs) with other pesticides and recommends eliminating problematic tank-mix applications and reducing fungicide and IGR applications during bloom [63]. However, an assessment of the California pesticide use data for San Joaquin county as a representative almond-growing region shows that progress in protecting honey bees from pesticide exposure is mixed.

Between 2014 when this study was conducted and 2022 (the latest year for which pesticide use data are available), overall fungicide use per bearing acre (BA) of almonds increased from 3.05 lbs/BA in 2014 to 4.16 lbs/BA in 2022 (see Supplementary Materials, Table S2).

For example, for the azole fungicides, use per bearing acre increased from 0.16 lbs/BA in 2014 to 0.20 lbs/BA in 2022. Applications during February (the month when almond pollination occurs) decreased slightly, from 2122 lbs over 12,411 acres to 1679 lbs over 11,320 acres, a decrease in application rate from 0.17 lbs/acre treated to 0.15 lbs/acre treated.

Similarly, application of strobil fungicides almost tripled, from 0.10 lbs/BA in 2014 to 0.27 lbs/BA in 2022, with the application rate during February remaining constant at 0.10 lbs/treated acre, but with the absolute amount of strobil fungicides used in February more than tripling, from 502 lbs over 5280 acres to 1604 lbs over 16,050 acres.

Use of the insect growth regulator methoxyfenozide increased from 6445 lbs total in 2014 to 21,599 lbs in 2022; however, use during the month of February decreased substantially, with the absolute number of acres treated in February decreasing dramatically from 5092 acres to 100 acres. Application rates were lower as well, from 0.18 lbs/treated acre in 2014 to 0.14 lbs/treated acre in 2022.

These mixed results indicate that grower education and outreach have the potential to mitigate pollinator exposures to problematic pesticides, but more needs to be done to raise awareness of the potential hazards of certain pest management practices to growers

and beekeepers. More effective would be for US EPA to require pesticide label changes prohibiting applications of problematic pesticides during bloom and to limit the practice of tank-mixing and co-application of pesticide chemicals. Further laboratory work to more clearly characterize problematic pesticide combinations is urgently needed.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14112505/s1>, Table S1: USDA Lab Analytes for the Study, including LODs, Current US EPA Registration Status, Use Type, and Chemical Classification; Table S2: Comparison of Pesticide Use on Almonds in San Joaquin County between 2014 and 2022^a.

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Conflicts of Interest: Author Jeff Anderson was employed by the company California-Minnesota Honey Farm. Author Darren Cox was employed by the company Cox Honey of Utah. Author Steve Ellis was employed by the company Old Mill Honey Farm. Author Geoffrey Marcy was employed by the company Space Laser Awareness. All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Colony Selection and Measurements

Appendix A.1. Colony Selection

Colonies selected for the study contained on average nine frames of bees with a standard deviation of 2.6 frames, based on the conventional frame-count method used in contracts for commercial pollination to assess hive strength (see below). Colony strength was assessed by counting the number of frames in each colony approximately 7–10 times (depending on beekeeper) during the period from January 2014 to January 2015, as described in the following section. Pollen, wax, and honey were sampled for pesticide analysis four times during the year. Pesticide analyses were focused primarily on pollen samples, with representative honey and wax samples analyzed at each sampling date.

The initial assessment of colony strength and chemical concentrations was made at the end of January 2014, prior to the placement of the hives in almond orchards for almond pollination. These measurements established the initial hive conditions, including the concentration of pesticide residues, the presence of pathogens and *Varroa* mites, and colony strength. The second sampling was conducted in late February and early March, immediately after the hives were removed from almond orchards. Two additional samplings of hive matrices to determine pesticide concentrations in the hives were conducted for each beekeeping operation. For the JA operation, the third sampling occurred in early April after cherry pollination and the fourth in late August. After almond pollination, the SE colonies were moved to the California foothills, and the DC colonies were move directly to

Utah. For both DC and SE operations, the third and fourth sampling dates were in early June and late August.

Throughout the year, beekeeping practices that may have affected colony health were monitored, including colony location, colony manipulations performed by beekeeper (i.e., splitting colonies, requeening), colony inputs (i.e., feeding sugar and protein patties, application of miticides or antibiotics), and queen performance and/or events (i.e., death, supersedure, fecundity). In addition, blooming crops near each apiary were noted. Lastly, observations such as unusual bee behavior, symptoms of disease, failure to thrive as expected for the season, the presence of dead bees at the hive entrance, and colony death were also recorded.

Appendix A.2. Colony Characteristics and Maintenance

Beekeepers DC, JA, and SE followed generally standard practices for migratory commercial beekeeping operations, including feeding sugar syrup and pollen patties as needed and administering treatments for *Varroa* mites and foulbrood throughout the course of the year. The DC and JA colonies were requeened when the absence of a queen or a failing queen was noted; the SE colonies were allowed to requeen themselves. Separate apiary locations were used by each beekeeper, with each pollinating a different almond orchard in Central California (with one exception—see below) and each then traveling to a unique summer location in Minnesota (JA and SE) or Utah (DC). Specifics for each beekeeping operation are described below.

Appendix A.3. DC Operation

The DC operation is based in northern Utah, in an agricultural area where alfalfa, small grains, safflower, mustard, fruit trees, pumpkins and melons are grown. Bees also forage on native plants, as well as dandelions, thistles, rabbitbrush, gumweed, wild sunflowers and other weeds.

The DC study hives were moved as follows:

- October 2013 to Oakdale and Hughson in California to overwinter.
- Early February 2014 to an almond orchard off of Victory Road near Escalon, CA for pollination.
- Mid-March to northern Utah after almond pollination was completed.
- 18 November 2014 to Oakdale and Hughson in California for overwintering.

Italian race bees were used, with queens purchased from Jackie Park-Burris Queens, Inc. and installed between April and July 2013. Only a single colony was requeened during the study, on 3/24/14. A total of 19 of the 20 colonies were split on 4/20/14, with four frames removed and the original queen left with the colony.

Bees were fed sucrose syrup, corn syrup, and pollen patties as needed. Mite treatments (Taktic, amitraz) were administered on 5/5/2014, 8/29/14, and 9/29/14. Treatment with terramycin as a prophylactic for foulbrood was administered on 9/29/14.

Appendix A.4. JA Operation

The JA operation is based in North Central Minnesota in the summer and fall, in an agricultural area where corn, soybeans, alfalfa, oats, barley, sunflowers and additional minor crops are grown. Bees also forage on native plants, tree pollens, dandelions, Dutch white clover, Canadian thistle, yellow and white sweet clover, goldenrod, birdsfoot trefoil and other weeds. The JA study hives were moved as follows:

- November 2013 to Oakdale and Copperopolis in California to overwinter.
- Early February 2014 to an almond orchard off McHenry Road near Escalon, CA for pollination.
- Early March 2014 to a cherry orchard off Copperopolis Road near Stockton for cherry pollination.
- Early April 2014 to Milton, CA.

- Early June 2014 to Eagle Bend in North Central Minnesota.
- 24 November 2014 to California near Copperopolis and Milton for overwintering.

Russian and Carniolan race bees were used, with queens either grafted from larvae taken from other hives in the JA apiaries and Lowman Apiaries, installed in late spring 2013. On 6/10/2014, all 15 colonies remaining in the JA group were found in crisis, with evidence of supersedure cells and dead bees in most colonies. At this point, all 15 colonies were requeened with queens donated by Lohman Apiaries. Only one of the twenty JA colonies was split on 5/26/14, with nine frames removed and the original queen left with the colony.

Bees were fed sucrose syrup and pollen patties as needed. Mite treatments were administered on 4/21/14 (Taktic, amitraz), 9/4/14 (Taktic, amitraz), and 10/20/14 (oxalic acid drench). Treatment with tylosin as a prophylactic for foulbrood was administered on 6/1/14.

Appendix A.5. SE Operation

The SE operation is based in West Central Minnesota in the summer and fall, in an agricultural area where corn, soybeans, alfalfa, sugar beets, edible beans, sunflowers and additional minor crops are grown. Bees also forage on native plants, tree pollens, dandelions, Dutch white clover, Canadian thistle, yellow and white sweet clover, goldenrod, birdsfoot trefoil and other weeds. The SE study hives were moved as follows:

- November 2013 to Angel's Camp in California to overwinter.
- Early February 2014 to an almond orchard off McHenry Road near Escalon, CA (SE-1 and SE-4) or to an almond orchard near Madera (SE-2, SE-3, and SE-5) for pollination.
- Late March 2014 to a non-agricultural area in Vallecito, CA in the Sierra foothills, where manzanita and other spring wildflowers were blooming.
- 19 April 2014 to an apiary in an agricultural area between Elbow Lake and Barrett, in West Central Minnesota.
- 23 November 2014 to California and overwintered in Angel's Camp, CA.

Russian and Carniolan race bees and survivor stock were used, with queens from Strachan Apiaries and Lowman Apiaries, installed in late spring 2013. Fifteen of the nineteen remaining colonies were split on 4/10/14, with nine frames removed and the original queen left with the colony. A second split of 9 frames on the same 15 colonies was performed on 5/23/14. On 7/11/2014, all 19 colonies remaining in the SE group were found in crisis, with evidence of supersedure cells. Supersedures were allowed to proceed naturally. No outside queens were introduced.

Bees were fed sucrose syrup and pollen patties, as needed. Mite treatments were administered on 2/1/14 (amitraz), 8/21/14 (oxalic acid drench), 9/9/14 (amitraz), and 10/24/14 (formic acid, half-dose). Treatment with Tylosin as a prophylactic for foulbrood was administered on 9/9/14.

Appendix B. Measuring the Bee Population Using "Frame Counts"

Appendix B.1. Frame Counts as a Metric of Colony Health

A metric of hive health was adopted based on the number of frames within a hive having a minimum number of bees [54,55]. A frame merited one "count" if 2/3 of both sides were covered by bees and/or brood. Photographs of the front and back of each frame were taken during each sample collection to provide a visual data archive, later evaluated by just one team member (RR) to reduce variation in assessments across beekeepers in determining whether the 2/3 criterion was met. This technique incurs the uncertainty of human assessment of "2/3", but it does not inject bias from hive to hive, beekeeper to beekeeper, or over time. In particular, differences in the number of frame counts over time offer a robust metric of changes in colony health. For each colony, frames were counted at each of approximately eight visits (depending on beekeeper) throughout the year, with results given in Tables A1–A3 (below).

Appendix B.2. Handling of Colony Splits

A total of 35 of the 60 colonies were split in the spring of 2014. For colonies that were split, the old queen was left with the original study hive, so there was no break in the brood cycle. The split was moved away from the study hive pallets and was no longer considered part of the study, except as described here. Frame counts from splits were accounted for by calculating a split factor (SF), defined as the number of frames remaining in the original hive after the split divided by the total number of frames in the original hive, e.g., for an 18-frame colony from which a 4-frame split was made, $SF = 14/18 = 0.78$. Corrected frame counts were calculated by dividing the measured frame counts by SF for all frame count measurements made after the split. This correction assumes that the population of the split changes at the same rate as the original colony. Many of the SE hives were split twice, necessitating the use of a second split factor, SF_2 , defined as the number of frames remaining in the split hive after the second split divided by the total number of frames in the original hive. The corrected frame count was calculated as the measured frame count divided by the product of SF and SF_2 .

Table A1. Frame counts over time for beekeeper DC.

DC Pallet#	Day 36	Day 73	Day 110	Day 168	Day 239	Day 273	Day 282	Day 401
1	27	51	68	77	73	53	34	9
2	41	44	51	63	65	72	56	15
3	43	65	68	71	60	39	19	5
4	35	54	68	84	88	81	59	19
5	41	49	68	78	100	82	73	23

Table A2. Frame counts over time for beekeeper JA.

JA Pallet	Day 28	Day 62	Day 97	Day 161	Day 245	Day 280	Day 395
1	27	58	64	66	52	16	4
2	30	56	54	51	30	9	7
3	34	52	68	61	28	18	15
4	47	60	67	68	34	15	8
5	34	53	53	43	26	22	13

Table A3. Frame counts over time for beekeeper SE.

SE Pallet#	29	68	100	122	143	160	192	237	289	395
1	43	49	53	101	103	118	75	133	79	42
2	35	48	61	113	109	124	84	55	49	18
3	38	46	64	134	128	152	120	100	16	4
4	41	42	48	92	96	124	120	116	20	12
5	38	42	60	96	94	132	150	162	12	15

Appendix C. Pesticide Data

Appendix C.1. Pesticide Concentrations, Limits of Detection, and Criteria for Inclusion

Overall, we found 37 chemicals, listed in Table A4, that exhibited at least one measurement significantly above the LOD. Ten chemicals had either three or fewer significant detections across all samples, namely, captan, cypermethrin, flubendiamide, myclobutanil, pyrimethanil, pyriproxyfen, spiroticlofen, tebufenozide, tetrahydrophthalimide, trifloxystrobin, and trifluralin. We retained these ten chemicals with their trace detections in the analysis that follows, but their integrity and statistical value remain unclear, and they are not included in the major results described below in Figures 2–4. Table A4 contains the sum of chemical exposure across the year for each of the 15 pallets for each of the 37 detected

chemicals. Pesticides with measured concentrations at or near the LOD or with three or fewer detections across the 15 pallets were not included in the statistical analysis, including aldrin, bifenthrin, chlorferon, DDE, endosulfan II (beta), fenpropathrin, fenpyroximate, metalaxyl, norflurazon, phorate, quinoxifen, sethoxydim, and tebuconazole.

Table A4. Sum of four measurements (ppb) of 37 chemicals for each pallet.

Pesticide	DC-1	DC-2	DC-3	DC-4	DC-5	JA-1	JA-2	JA-3	JA-4	JA-5	SE-1	SE-2	SE-3	SE-4	SE-5
2,4-Dimethylphenyl formamide	90.2	68.7	34.3	47.3	63.7	0.0	24.4	28.1	35.2	61.3	0.0	0.0	0.0	151.0	0.0
Acetamiprid	0	0	0	0	0	0.77	0.76	0.92	0	0	0	0	0	0	0
Atrazine	0.0	0.0	0.0	0.0	0.0	50.1	17.1	0.0	222.0	76.2	0.0	0.0	0.0	0.0	0.0
Azoxystrobin	0.0	0.0	0.0	0.0	0.0	10.4	0.0	107.6	16.5	8.7	0.0	0.0	0.0	0.0	0.0
Boscalid	0.0	0.0	0.0	0.0	0.0	908.4	844.0	726.7	572.2	715.7	21.4	128.0	22.7	19.1	28.1
Captan	10.0	10.0	10.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Carbendazim	5.0	0.0	0.0	5.0	5.0	16.2	17.9	12.7	16.5	11.8	8.4	124.0	94.6	18.8	111.0
Chlorothalonil	1.0	2.0	3.0	1.0	2.0	2.0	2.0	2.5	2.5	2.5	197.0	98.5	2.0	1.0	2.0
Chlorpyrifos	298.0	118.0	272.9	158.4	156.9	132.5	75.2	166.7	94.3	70.1	30.8	25.7	22.0	43.2	32.4
Clothianidin	0	0	0	0	0	0	0	0	0	0	17.30	22.94	4.46	35.73	11.20
Coumaphos	6.0	75.8	62.1	28.3	22.6	16.8	91.4	15.9	122.9	17.3	14.6	9.4	6.9	12.5	92.3
Coumaphos oxon	0.0	5.0	0.0	0.0	0.0	0.0	6.2	0.0	5.0	0.0	5.0	0.0	0.0	0.0	0.0
Cyhalothrin, lambda	17.9	13.4	19.1	24.7	14.3	2.0	0.0	3.0	0.5	0.0	18.3	14.9	11.5	21.4	18.5
Cypermethrin	0.0	0.0	0.0	0.0	0.0	0.0	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cyprodinil	32.5	12.8	13.2	10.2	15.5	322.4	207.2	245.1	132.7	413.0	476.0	20.1	27.4	271.0	12.5
Diflubenzuron	0.0	0.0	0.0	0.0	68.9	0.0	0.0	0.0	0.0	0.0	163.0	213.0	294.0	209.0	717.0
Dinotefuran	2.5	1.8	1.1	3.0	2.1	0.0	1.6	1.1	1.1	2.3	1.6	1.8	0.0	1.3	1.1
Esfenvalerate	0.0	0.0	0.0	0.0	0.0	9.1	0.0	1.0	3.0	1.0	2.1	2.0	2.0	4.9	2.0
Etoazole	0.0	0.0	1.0	1.4	0.0	15.2	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fenbuconazole	0.0	0.0	0.0	0.0	0.0	50.2	160.3	227.9	144.6	22.5	0.0	0.0	10.0	14.4	0.0
Flubendiamide	0.0	863.0	629.0	182.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Imidacloprid	2.7	2.0	3.2	1.7	2.9	3.0	4.9	5.8	4.9	5.5	3.9	4.5	2.7	4.9	2.7
Methoxyfenozide	63.4	314.0	159.2	104.0	95.5	361.0	551.0	532.0	483.5	323.1	127.0	78.3	47.8	64.5	41.3
Myclobutanil	0.0	0.0	0.0	0.0	0.0	62.5	0.0	0.0	36.2	35.0	0.0	0.0	0.0	0.0	0.0
Oxyfluorfen	0.0	65.1	4.0	0.0	37.4	66.3	0.0	29.5	0.0	0.0	0.0	0.0	0.0	0.0	19.8
Pendimethalin	107.0	113.0	172.4	104.4	196.6	837.4	399.0	397.0	428.0	176.6	117.0	247.2	176.0	160.0	156.0
Pyraclostrobin	0.0	0.0	0.0	0.0	0.0	591.3	597.0	559.0	387.0	464.0	19.7	181.0	22.3	15.2	22.9
Pyrimethanil	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0	5.0	0.0	3.8
Pyriproxyfen	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	11.0	0.0	0.0	7.2	0.0	0.0	5.5
Spirodiclofen	0.0	0.0	5.0	0.0	4.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tau-fluvalinate	13.1	15.7	85.5	36.6	26.0	2.8	0.0	9.5	19.8	7.9	16.1	8.1	15.4	15.8	22.1
Tebufenozide	0.0	0.0	0.0	0.0	0.0	0.0	29.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tetrahydrophthalimide	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thiamethoxam	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.24	1.18	0.53	0.71	0.00
Thymol	0.0	0.0	0.0	100.0	50.0	150.0	100.0	616.0	839.5	236.0	1330.0	651.0	1650.0	1057.0	2548.0
Trifloxystrobin	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	57.0	0.0	0.0	0.0	0.0	0.0
Trifluralin	2.0	2.0	2.0	3.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	3.0	3.0	2.0

Appendix C.2. Variability in Pesticide Concentrations Across Pallets

Although pallets from each beekeeper were placed in different almond orchards in February (with the exception that SE-1 and SE-4 were placed with the JA pallets), the mix of pesticides found in the post-almond pollination samples from each beekeeping operation was similar, dominated by pesticides applied during almond bloom. Analysis of the individual hive samples allowed for an assessment of the variability of pallet pesticide measurements in pollen across hives in the same location and in different locations. Figure A1 shows the variability in concentrations of a commonly detected pesticide, cyprodinil, over the different hives on a pallet and across the different pallets for each operation. The data indicate that while concentrations may vary by a factor of up to 11 across individual hives, pallet concentrations only vary by up to a factor of 3.6.

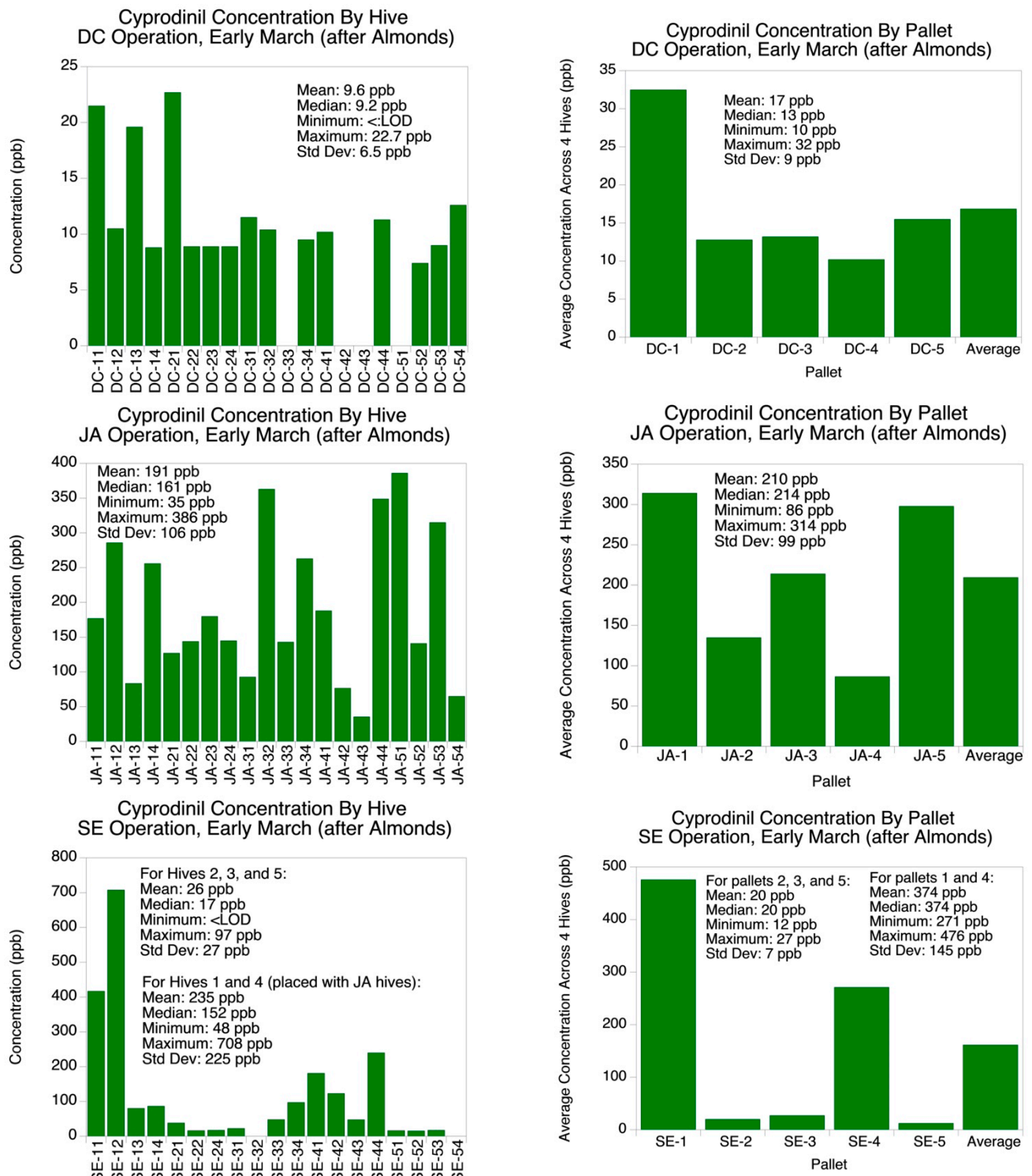


Figure A1. Variability in pesticide concentration across hives and pallets for different beekeeping operations. Note that pallets SE-1 and SE-4 were placed with the JA pallets instead of with the rest of the SE hives; thus, the statistics were calculated separately.

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