ABSTRACT

Title of Thesis: PESTICIDE RESIDUES IN BEESWAX PREDICT THE OCCURRENCE OF VARROA WITH ALLELES THAT CONFER AMITRAZ RESISTANCE.

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Honey bees are important pollinators and are essential to modern industrial agriculture. One of the largest threats to honey bee health is the parasitic varroa mite *Varroa destructor*. A common method beekeepers use to control varroa is the application of synthetic varroacides. Following years of treatment, varroa have developed resistance to multiple varroacides leading to treatment failures. This project set out to examine the relationship between pesticide residues within beeswax and resistance alleles in varroa. I expected that the presence of varroacides would be positively related to the occurrence of known mutated alleles that confer varroacide resistance to varroa. We looked for 3 different known varroacide resistance alleles, and only found 2, (N87S and Y215H). Both N87s and Y215H confer resistance to amitraz and her metabolite DMPF. Only one mutation, the Y215H mutation, was widespread, occurring in 68% of the 195 mites we examined. We found that the mutation occurred more frequently in mites that were collected from apiaries that had higher DMPF levels. Surprisingly, the presence of other unrelated pesticides (e.g. not having the same mode of action as amitraz) were also positively correlated with the proportion of mutated alleles found. Both the total number of pesticides found in an apiary, and increasing concentrations of fungicides, insecticides, and varroacides (including and excluding DMPF data), predicted higher odds of finding the Y215H mutation. It is unclear if this relationship is a result of a correlation between pesticide levels, if the mutation help benefits the mites resist pesticide more generally, or if the gene has become fixed in the population. It is important to monitor resistance conferring mutations in the varroa mite population in order to help beekeepers make proper varroa management decisions.

PESTICIDE RESIDUES IN BEESWAX PREDICT THE OCCURRENCE OF VARROA WITH ALLELES THAT CONFER AMITRAZ RESISTANCE.

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Table of Contents

List of Tables

List of Figures

Introduction

The western honey bee (*Apis mellifera*) is an important part of industrial agriculture in the United States. Large scale crop production requires insect pollination services, which are mainly provided by managed honey bees. Honey bee pollination services in the United States (US) are valued at $16 billion USD annually (Losey and Vaughan 2006). Understanding the drivers of managed honey bee health within the US is important in mitigating losses of managed honey bees, allowing them to continue their role within the US as an important pollinator.

Varroa destructor as a serious threat to honey bees

One of the parasites monitored by the National Honey Bee Survey is the varroa mite (*Varroa destructor*). Varroa are considered the greatest threat to honey bee health worldwide (Traynor et al. 2020). Varroa reproduce quickly within a honey bee colony requiring monitoring to ensure populations have not grown unexpectedly (Rosenkranz et al. 2010). Varroa feed on hemolymph and the fat body tissue of immature and adult honey bees (Ramsey et al. 2019). In addition to the direct effects of parasitism, varroa vector viral diseases such as Deformed Wing Virus A (DWV-A) (Posada-Florez et al. 2020). These factors make varroa a consistent driver of honey bee losses (Nazzi et al. 2012, Rosenkranz et al. 2010). Originally a parasite of the Asian honey bees (*Apis cerana*), varroa mites were first seen parasitizing Western honey bees (*Apis mellifera*) in Japan in 1957. In the 1970s varroa mites spread across the world assisted by shipping and trade. As a novel host, *A. mellifera* populations do not have the evolved responses to mitigate varroa without pest management interventions.

Many different varroa management methods have been developed. Synthetic miticides, (e.g. amitraz, (formamidine that targets octopamine receptors), coumaphos, (organophosphate

that targets acetylcholinesterase) and fluvalinate, (pyrethroid that targets the voltage-gated sodium channels) are miticides that have been registered for use against varroa. Collectively we call these products varroacides. Varroacides can have negative impacts on honey bees, with evidence of elevated expression of genes related to stress and detoxification in colonies exposed to chemical varroa treatment (Boncristiani et al. 2012); however, these effects are less detrimental than those caused by mites' population when they are left untreated. Chemical treatments help to control varroa mite loads and limit the damage they cause to honey bee colonies. In the US, after the initial invasion of varroa, products like fluvalinate and coumaphos were used excessively and exclusively leading to high levels of resistance in varroa populations (Kange et al. 2010, Rinkevich 2020). Resistance to fluvalinate was first noticed from field treatment failures (Thompson et al. 2003) and became widespread in the US (Millán-Leiva et al. 2018,). While initially registered for use, the now common pesticide, amitraz, was withdrawn from the market over concerns of damage to queen health, only to be reintroduced after the other widely used product for varroa control began losing effectiveness. Fluvalinate, a pyrethroid, acts on the sodium ion channel of susceptible mites. When random mutations occur within genes that encode the sodium ion channel, mites can become resistant to fluvalinate and other pyrethroid pesticides that act on this same target (González-Cabrera 2013). Mutations in the oxidative detoxification mechanism within varroa have led to resistance to the organophosphate varroacide coumaphos (Li et al. 2003, Kanga et al. 2010). Similarly, a mutation in the octopamine receptor beta-2R-like confers resistance to amitraz (Hernández-Rodríguez et al. 2022). Resistance often has the tradeoff of lower fecundity within varroa making resistant varroa less fit in environments without varroacides present. In environments with lower varroacide levels, there is selection pressure against resistant mutants (González-Cabrera et al 2016).

Widespread resistance presents a serious problem as chemical miticide treatments are used as all or part of varroa management by approximately 80% of small-scale beekeepers and more than 90% of large-scale beekeepers (Haber et al. 2019). Given that 65% of large-scale beekeepers report only using chemical control to manage varroa (Haber et al. 2019), understanding the effect pesticide residues may have on selecting for varroa resistance may have important implications for future varroa management.

Current varroa mite management

Managing varroa is an essential part of modern bee management (Rosenkranz et al. 2010, Giacobino et al. 2016). Varroa management tools include natural and synthetic chemical treatments. Natural chemical treatments include organic acids, formic acid (Formic-pro), oxalic acid (Api-bioxal) and thymol (Apilife VAR, Api-guard). Common synthetic treatment options available in the US include amitraz (Apivar), coumaphos (Mite Check), and fluvalinate (Apistan). All of these treatments are either volatile or lipophilic to avoid honey contamination (Rosenkranz et al. 2010, Albero et al. 2023). Chemical treatments can be an effective strategy for managing varroa mite load; using only non-chemical treatments alone, without use of some chemicals, are associated with the higher losses for beekeepers (Haber et al. 2019) Chemical treatments are often applied multiple times a year, with applications timed according to nectar flows and mite population growth (Abou-Shaara 2014). However, while such timing also helps avoid honey contamination, lipophilic compounds, will accumulate within wax, potentially leading to long term exposures (Sanchez-Bayo & Goka 2014). Non-chemical management options include a brood break, created by caging or introducing a new queen thereby making the colony brood less and preventing mites from reproducing. As there are no mite reproducing in capped cells during this time, the mites are more vulnerable to honey bee grooming behavior and

other management practices designed to remove mites from adult bees (Seeley & Smith 2015). Selectively breeding bees more able to control or tolerate mites and their vectored disease also hold promise (O'Shea-Wheller et al. 2022). While a long term and sustainable solution to varroa is the widespread adoption and use of varroa resistant or tolerant honey bee stock, these stocks are not yet widely available or are not suitably productive for commercial beekeepers. Many non-chemical control methods are time intensive and so are practices that maybe suitable for smaller scale beekeepers, they are unsuitable for larger scale commercial and migratory beekeepers who are more reliant on cost effective chemical treatments (Rosenkranz et al. 2010).

Field evolved resistance

Field evolved resistance is a genetically based decrease in susceptibility of a target population to a toxin caused by exposure to the toxin in the field (Sumerford 2013). As a chemical control is used consistently over time, mutations that confer resistance to the chemical treatment are more likely to spontaneously occur, be selected for, and spread in the pest population (Siegfried and Hellmich 2012). Mutations in key genes, such as the sodium-gated ion channel that is targeted by pyrethroids, reduces an individual's likelihood of mortality after chemical exposure, then these mutations are expected to spread in a population when that population remains exposed (González-Cabrera et al. 2013). However, such mutations can have ill effects on a mutated individuals' fitness, and so would be selected against in unexposed populations (González-Cabrera et al. 2013). Continued use, or exposure, to the same chemical pesticide treatments allows resistance to develop, spread and become stable in pest populations. Rotating chemical treatments, however, can help break this selection cycle, and prolong the usability of chemical treatments (Vandervalk et al. 2014).

Beeswax as a matrix for pesticide residue

Foraging honey bees bring back field contaminated pollen and/or nectar, is one route of pesticide exposure in honey bee colonies. Application of varroacide directly into the colonies is another. Of the many types of chemicals that can accumulate in honey bee colonies, lipophilic varroacides are often the most prevalent and abundant due to their high affinity with beeswax and stability within hive temperature conditions (Lozano et al. 2019). Residue accumulation within beeswax exposes mites to low levels of products over a long period of time (Tosi et al. 2022). Studying relationships between pesticide residues in colonies and the rate of known varroacide conferring resistant mutations with US varroa populations could help inform the future direction of varroa management research and practice.

Metrics for measuring pesticide exposure

Pesticide exposures can negatively impact honey bee health (Goulson et al. 2015). A single pesticide or combination of pesticides can have measurable impact on colony health even if exposures are below lethal levels (Tosi & Nieh, 2019). Sublethal effects, by definition, do not cause individual bee mortality, they can negatively impact the colony (Tosi et al. 2022). Previous studies have quantified pesticide exposure differently in order to account possible synergistic or antagonistic relationships between products. Studies have looked at the effect of the total number of pesticides present in samples (Tosi & Nieh 2019, El Agrebi et al. 2020, Traynor et al. 2021) the total amount of pesticides within a sample, measured as ppb (or ng/g), as a measure of pesticide exposure. While these analyses have proven valuable in finding relationships between infield exposures and honey bee disease outcomes in colonies (Traynor et al. 2021, El Agrebi et

al. 2020), they fail to incorporate toxicity and the concentration with which it is found in samples (Sanchez-Bayo & Goka 2014).

Risk quotient (RQ) scores are calculated by dividing the exposure dose or concentration of a pesticide by its known lethal dose or concentration - either its LD_{50} (lethal dose that kills 50% of the treated population) or its LC_{50} (lethal concentration that kills 50% of the treated population). RQ scores for individual pesticides can be summed to calculate the total RQ score of a sample. In order to elucidate the risk associated with exposure to different classes or mode of actions (MOAs), RQ scores can be calculated for classes and each MOA (Carlson et al. 2022). One key limit of risk quotient is that when multiple pesticides are found together their combined effect is often assumed to be additive, while different pesticide combinations are known to have synergistic (e.g. Thiamethoxam & Permethrin) or antagonistic (e.g. Thiamethoxam & Esfenvalerate) effects (Li et al. 2023, Tosi et al. 2023). Honey bees act as a standard organism to study pesticide effects (US EPA OPP et al. 2014), and so LD and LC 50 are often readily available, however for organisms that do not require testing for product registration, these values are not always available. For this reason, RQ scores for an organism like varroa are only available when experimentally determined throughout the course of previous published research studies.

This study set out to look for a relationship between the frequency of varroacide resistant alleles an apiaries varroa population and the amount of fluvalinate and amitraz residues that were found in honey bee colony brood wax from those same apiaries. Fluvalinate is stable in wax (Frison et al. 1999) and can remain stable for over 8 months at hive conditions (Tsigouri et al. 2001). We expected to see higher concentrations in operations that had a history of using the product, and so remaining mites in these operations would be more likely to carry fluvalinate

conferring resistance genes. We also used the same logic to develop a hypothesis concerning mite resistant alleles and amitraz residues in comb wax. On account of amitraz's short half-life (1 day in beeswax) (Korta et al. 2001), we used the amitraz metabolite N-(2,4-dimethylphenyl) formamidine (DMPF), which is stable in beeswax, as a proxy for amitraz exposure. DMPF acts in a similar way to its parent compound (Takata et al. 2020). With a LC_{50} of 0.497 µg/ml (Morsy et al 2022), we expect DMPF to select for resistant alleles in the same way as the parent product. In short, I hypothesized that the varroa remaining in colonies that had high levels of DMPF in comb wax would be more likely to carry amitraz resistance conferring genes.

While I did expect to see a positive relationship between the presence and number of products (amitraz and fluvalinate) that had resistant alleles, and resistant conferring genes in varroa, I expected this trend to only extend to resistant pesticides. I did not expect levels of other non-varroacide products, specifically those from different pesticide classes, to predict the frequency of resistant genes found in sampled varroa. These hypotheses were tested by looking for a relationship between our different measures of exposure (presence, count, concentration) in apiary level brood wax samples and the frequency of resistant genes found in mites collected from those same apiaries.

Methods

Sample collection

The National Honey Bee Health Survey (NHBS) is a cross sectional survey of managed honey bee colonies in the US. Funded by the USDA Animal Plant Health Inspection Service (APHIS), the NHBS is used to survey threats to honey bee health, including quantifying levels of disease and risk exposures (https://ushoneybeehealthsurvey.info/). The NHBS monitors honey

bee colonies for a select group of known threats to honey bee health including disease, pesticide, and pests impacting honey bees in the US. Samples include adult bees for viral and parasite quantification, and, in some cases either bee bread (stored pollen) or comb nest wax for pesticide residue analysis.

Forty-one states or territories (including Guam, and Washington D.C.) were sampled by the NHBS over the 2022-2023 NHBS sampling year. As part of the standard NHBS sampling, nurse bees from all sampled colonies (approximately 4 oz or 300 adult bees per colony) were collected and stored in alcohol and refrigerated until shipped to the Honey bee Lab at the University of Maryland College Park. Of these, nine states/territories (California, Colorado, Washington D.C., Delaware, Michigan, Minnesota, New Mexico, Washington, and Wisconsin) had sampling teams that agreed to send an additional 3-gram brood wax sample along with the usual NHBS samples of bees and bee bread taken for pesticide analysis. Apiary Inspectors who did collect brood nest wax samples were instructed to collect wax samples from the same (4 to 8) colonies from which NHBS samples were derived. Inspectors were asked to remove wax samples using hive tools by cutting a quarter turn (90°) section of wax from a brood frame in an area of drawn comb that held little resources (nectar, honey or bee bread) or bee brood. Wax taken from all eight sampled colonies was pooled into one 50-mL Falcon tube to serve as a single apiary level sample. Wax samples were kept out of sunlight and frozen until being shipped to the Honey bee Lab at the University of Maryland College Park where alcohol preserved bee samples with varroa were analyzed. Wax samples were frozen again and shipped to the USDA Agricultural Marketing Services (AMS) laboratory in Gastonia, North Carolina for pesticide analysis.

Alcohol-preserved bees were used to quantify varroa levels as well as provide an archived subsample for potential future research. The alcohol-preserved bees were shaken in alcohol to dislodge varroa mites that were on the bees. The bees and varroa were counted to provide an estimation of varroa load. For the 39 samples received for this study, collected varroa mites were stored in 70% ethanol in a 2ml microcentrifuge tube (Fisherbrand Premium 2 ml microcentrifuge 508GRDPFB) and frozen until processing.

Pesticide Analysis

Thirty-nine brood wax samples were sent for pesticide screening at the USDA National Science Laboratories Agricultural Marketing Services in Gastonia, North Carolina using the Apiculture Pesticide Screen (https://www.ams.usda.gov/services/lab-testing/nsl). Extraction and analyses were carried out according to the Apicultural Pesticide screen protocol. Detection methods used include gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) and liquid chromatography (LC-MS/MS). This process is able to detect 192 chemicals from pesticides or pesticide residues.

Varroa Genotyping

Samples that contained at least 5 mites and that were paired with wax samples were used for analysis. Five mites were selected at random from each sample and each mite was placed individually into a microcentrifuge tube. The mites were stored at -80 °C to preserve DNA prior to extraction. Mites were homogenized with 180 µL of ATL Buffer (tissue lysis buffer) (QIAamp DNA Micro kit, Cat. No. / ID: 56304) using a sterilized pestle in their microcentrifuge tubes. Each pestle was washed with 200µl of AL Buffer to ensure the entire sample remained when the pestle was removed. DNA was extracted from each sample using the QIAamp All-Prep DNA extraction protocol for small blood samples. To detect mutation of the Y215H and N87S

alleles that confer resistance to amitraz, TaqMan genotyping assay was used as described in Hernández-Rodríguez et al., (2022). Genotyping assay for the Mutation N87S included flanking primers “OctR_Vd_87_F” 5’-CGCCCTGTTCGCGATGA-3’ and “OctR_Vd_87_R” 5’-ATCCACTTGCCCGAAATGGT-3’, FAM-labeled probe for the mutant amitraz-resistant allele, FAM-5’-CGACGCACTGAATG-3’, and VIC-labeled probe for wild-type allele, VIC-5’-ACGACGCATTGAATG-3’.

To validate the assay, it was essential to use control oligonucleotides corresponding to WT and mutated targets:

>Contr-Vd_87-wt

CGCCCTGTTCGCGATGACATTCAATGCGTCGTTCACCATTTCGGGCAAGTGGAT

Contr-Vd_87-MUT

CGCCCTGTTCGCGATGACATTCAGTGCGTCGTTCACCATTTCGGGCAAGTGGAT

Genotyping assay for the Mutation Y215H included flanking primers “OctR_Vd_215_F”, 5’-GGATACCGTGCTCAGTAATGCT-3’’ and “OctR_Vd_215_R “, 5’-CTGTCGGGTCGCTTCTAGATAG-3’, 6-FAM-labeled probe for the mutant amitraz-resistant allele, FAM-5’-CGCCAATGAGTGAAT-3’, and VIC-labeled probe for wild-type allele, VIC-5’-ATGCGCCAATAAGTGAAT-3’.

To validate the assay, it was essential to use oligonucleotides corresponding to WT and mutated targets:

>Contr-Vd_215_wt

GGATACCGTGCTCAGTAATGCTATTCACTTATTGGCGCATCTATCTAGAAGCGACCCGACAG

>Contr-Vd_215_MUT

GGATACCGTGCTCAGTAATGCTATTCACTCATTGGCGCATCTATCTAGAAGCGACCC GACAG

To detect mutations in sampled varroa conferring resistance to fluvalinate TaqMan genotyping assay as described in Millán-Leiva et al. (2021). Genotyping assay for the Mutation L925I included flanking primers "VGSC-925-FLANK-For" 5'- CCAAGTCATGGCCAACGTT-3', and "VGSC-925-FLANK-Rev", 5'-AAGATGATAATTCCCAACACAAAGG-3', FAM-labeled probe for the mutant tau-fluvalinate-resistant allele L925I, FAM-5'-AGGTTACCTATAGCTCC-3', and VIC-labeled probe for wild-type allele, VIC-5'- AGGTTACCTATAGCTCC -3'. Genotyping assay for the mutation L925M included FAM-labeled probe for the mutant tau-fluvalinate-resistant allele L925M, FAM-5'- TTACCCATAGCTCCTATC-3', and VIC-labeled probe for wild-type allele, VIC-5'- TTACCCAGAGCTCC.

Data Handling and Statistical Approach

Pesticide residue data were obtained from the NHBS database (https://ushoneybeehealthsurvey.info/). The varroa resistance data were combined with the pesticide residue data to create a paired dataset of resistant vs. susceptible alleles and pesticide profiles. Specific LC_{50} values were obtained from the Pesticide Properties Database, University of Hertfordshire (Lewis et al. 2015 http://sitem.herts.ac.uk/aeru/ppdb/).

Pesticide detections were classified as trace level when above the limit of detection (LoD) but below the limit of quantification (LoQ). These values were considered positive for pesticide count data but excluded from concentration comparisons. Pesticides were categorized by class (fungicide, herbicide, insecticide, varroacide).

Pesticide prevalence (%) was calculated by dividing the number of positive detections (including trace) by the total number of samples (39) and multiplying by 100%. The number of

products found in each sample was calculated by adding the number of detections (including trace). The resulting count data was used to divide the resistance gene data set into quartiles in order to compare the frequency of each resistant allele between exposures. Comparisons were made using the chi-square or fisher's exact test (Stats package in R studio) Data were fit to a binomial GLM with quartile as the independent variable and the number of resistant alleles as dependent variable (considered either successful (mutant) or failure (wildtype). The model was created using the glm function as follows for total detections and concentration:

glm (formula = (ra, 10 - ra) ~ quartile, family = "binomial"

For pesticide class analysis the above model was only given pesticide data for one class at a time for analysis, and when analyzing the data with DMPF removed, samples DMPF ppb and detection data were removed from the dataset before modeling.

Log odds estimates calculated by the model were exponentiated to acquire odds ratios and their confidence intervals using the R stats package (4.4.0). We then repeated the analysis, grouping pesticides by class rather than analyzing all pesticides in each sample together. The same approach was used for the concentration (ppb) data.

The role of DMPF specifically was explored by considering if and how relationships between pesticide residues and resistance alleles in varroa changed when DMPF was included and excluded from analysis. The same concentration analyses were performed with the DMPF contribution to data removed to determine how much of the relationship was driven by DMPF. A t-test was performed to compare the mean resistance alleles in samples without DMPF to samples with DMPF. All statistical analyses were conducted in R version 4.2.1 (R Development Core Team, 2022).

Results

National Honey Bee Disease Survey field samplers provided 39 samples for this study from the following states/territories California(n=4), Colorado(n=2), Washington D.C.(n=8), Delaware(n=6), Michigan(n=3), Minnesota(n=3), New Mexico(n=6), Washington(n=2), and Wisconsin(n=5) (see Supplemental Table 1). The average varroa mite load in these apiaries was 3.52 ±0.82, range: 0.58 - 30.86 varroa/100 bees (see supplemental table 1). Five mites were randomly selected from each of the stored samples. These were individually processed to determine the presence of 3 different varroacide resistant alleles.

Resistance alleles

Resistance Allele	Resisted pesticide	Description
L925MET	Fluvalinate	Point mutation in the sodium-gated ion channel associated with pyrethroid resistance
N87S	Amitraz	Point mutation in the binding region of β-adrenergic-like octopamine receptor
Y215H	Amitraz	Point mutation in the binding region of β-adrenergic-like octopamine receptor

Of the three different resistance mutations examined, one confers resistance to fluvalinate (L925MET) (González-Cabrera et al. 2016) and the other two confer resistance to amitraz (N87S & Y215H) (Hernández-Rodríguez et al. 2022). All of these mutations are recessive, and so only mites with two copies of the same mutation are phenotypically resistant. The resistance allele for fluvalinate was not found in any of the mites analyzed. Three of 195 mites analyzed had a single copy of the N87S resistance allele. These three mites were found in samples from Colorado, Michigan, and Nevada. The Y215H mutation was widespread, with 132 (of 195) mites from 35 (of 39) apiaries having the Y215H resistance allele. Of the mutation-carrying mites, 121 were

homozygous mutants, while 11 were heterozygous for the mutant (and therefore phenotypically susceptible to treatment). Only the Y215H mutation was found with enough frequency to test our general hypothesis that frequency of resistance genes can be predicted by brood wax DMPF contamination as an indicator of amitraz levels.

Pesticide count and frequency of resistant alleles.

Brood wax samples were screened for 192 pesticides and/or metabolites. Fifty-five pesticides/metabolites were detected at least once. Eleven of these pesticides/metabolites were only ever detected at trace levels. All analyzed wax samples had at least one pesticide detected, although one sample from Delaware (DE-19-2022) only had trace contaminants of seven products detected (acetochlor, azoxystrobin, carbendazim, DEET, diuron, metolachlor, & piperonyl butoxide). DMPF, the metabolite of amitraz, was prevalent in 35 samples, and was the most frequently found contaminant (Table 1).

The average number of pesticides detected per sample (including trace detections) was 12.8±1.33 (range 1-38) (Table 1). To compare the frequency of the Y215H mutation in samples with different levels of exposure, samples were divided into 4 equally sized groups based on the rank order of the number of pesticides found. Varroa from apiaries that had higher numbers of pesticides in their brood wax also had a higher frequency of the Y215H resistant mutation (Fisher's exact test $p<0.007$ $n=247$ Figure 1a). The odds of finding resistant alleles in varroa mites dramatically increased in samples with higher and higher numbers of pesticides detected (Figure 2).

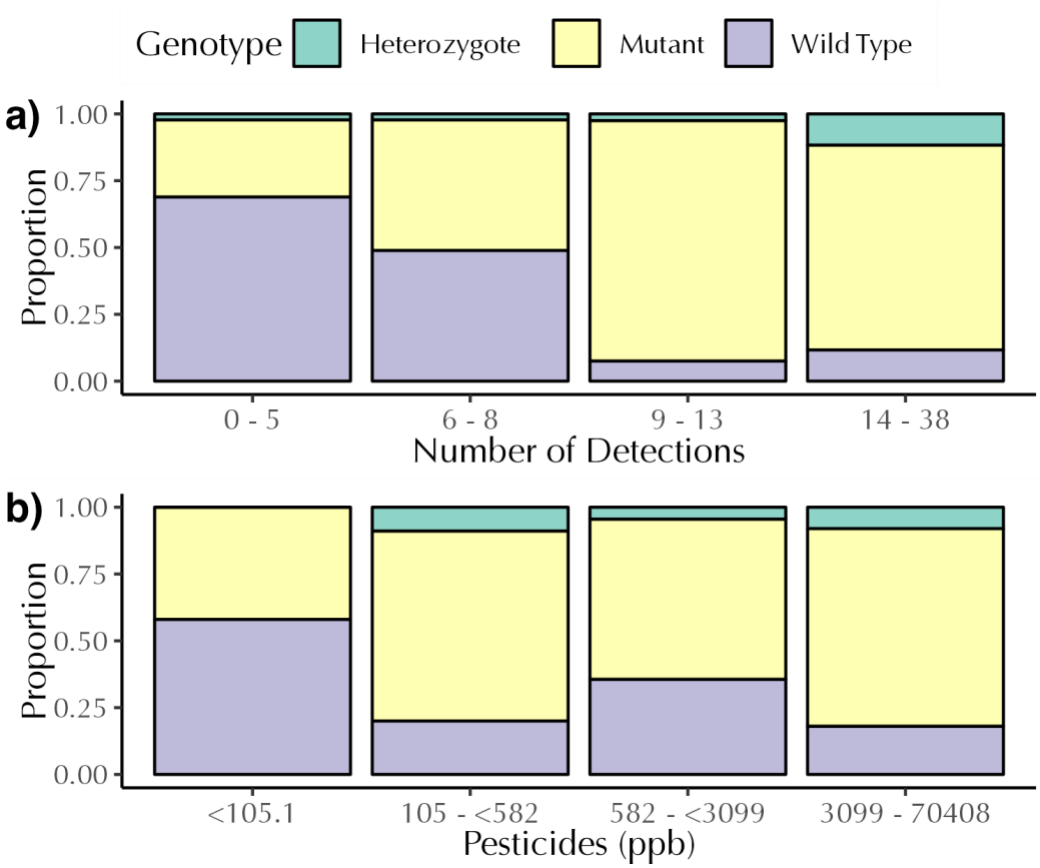

Figure 1. The proportion of mites that were homozygous wild type, heterozygous, or homozygous for the Y215H mutation and were from samples with different levels of pesticide risk exposure as measured by a) Count (The number of pesticides/metabolites detected in the sample b) concentration (ppb) of all pesticides in a sample combined.

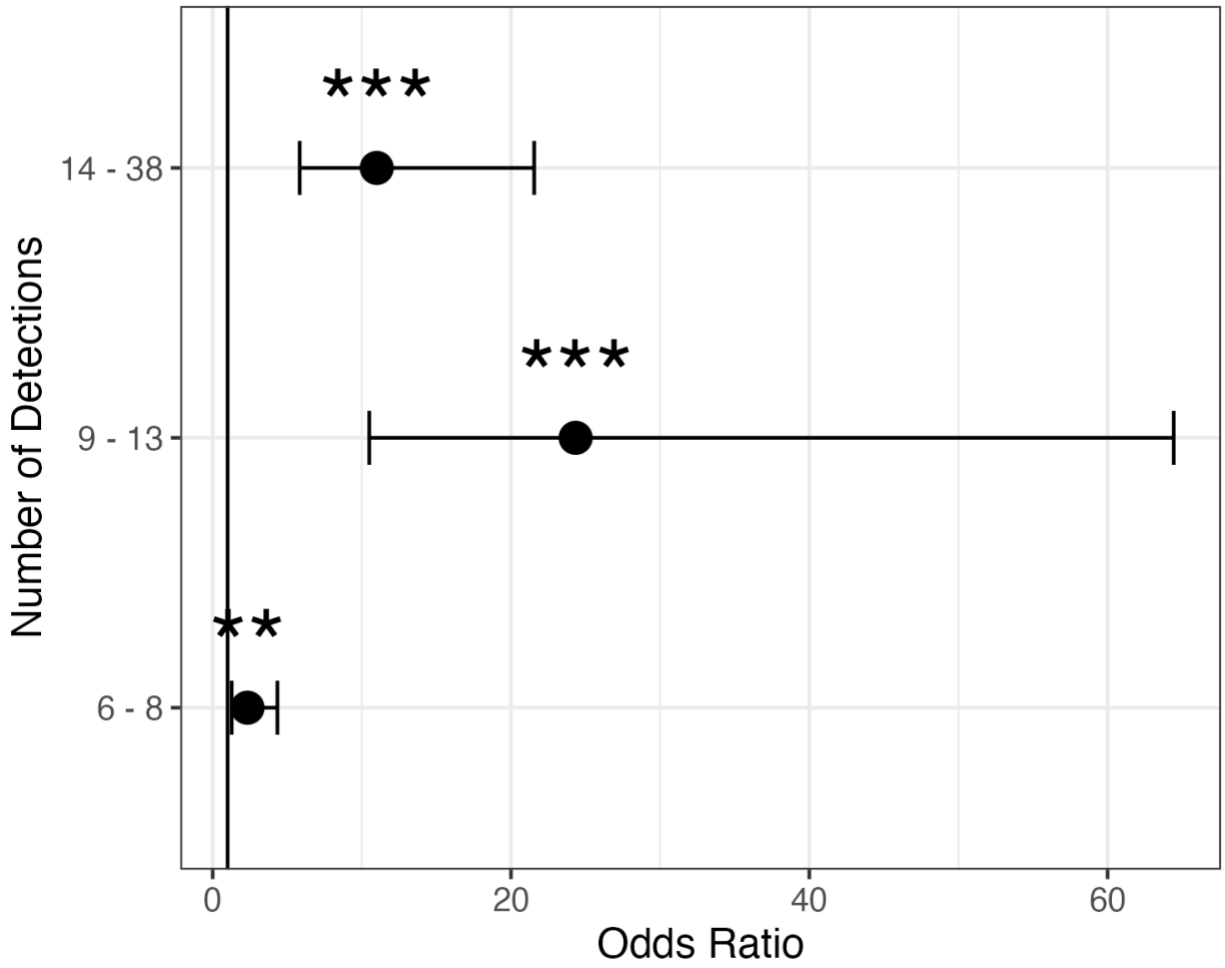

Figure 2. The Odds of detecting mutation Y215H in mites in apiaries with different counts of pesticides found in samples. Significantly increased odds, as compared to the lowest count quartile, are indicated by asterisks in apiaries that had higher counts of pesticides in samples. Significance codes: 0***, 0.001**, 0.01*.

Of the 55 pesticides/metabolites detected (Table 1), 22 were fungicides, 7 were herbicides, 21 were insecticides, and 5 were varroacides. On average, each sample had a total number of 5.48±0.76 fungicides, 1.54±0.13 herbicides, 0.48±0.46 insecticides, and 2.3±0.22 varroacide detections. Within all samples, fungicides were detected 252 times, herbicides were detected 71 times, insecticides 160 times and varroacides 106 times. While the frequency of resistant alleles did not change over the gradient of fungicides or herbicides detected (fungicides Z=0.5, p=0.61(figure 3e)) (herbicides Z=-0.3, p=0.77(figure 3d)), increasing numbers of

insecticides and numbers of varroacides in samples significantly increased the proportion of resistant varroa in a sample (insecticides Z=5.1, p<0.001(figure 3c) varroacide Z=2.7, p=0.007) (Figure 3a). When compared to the quartile of samples with the lowest number of insecticides and varroacides detected, all other quartiles (combined) had significantly higher odds of encountering Y215H mutations for insecticides (OR (95%CI) 1.72 (1.41-2.14), and varroacides 1.37 (1.09-1.73). This did not hold true for pesticides classified as fungicides 1.02 (0.93-1.12), or herbicides 0.95 (0.7-1.29). We examined the relationship between DMPF and resistance allele frequency. DMPF was found in a total of 35 samples and in 28 samples above the limit of quantification (Table 1). Fewer resistant varroa were found in samples that did not contain DMPF than those samples that contained DMPF when analyzed with a t-test (t=-2.78 df=13.5 p=0.015).

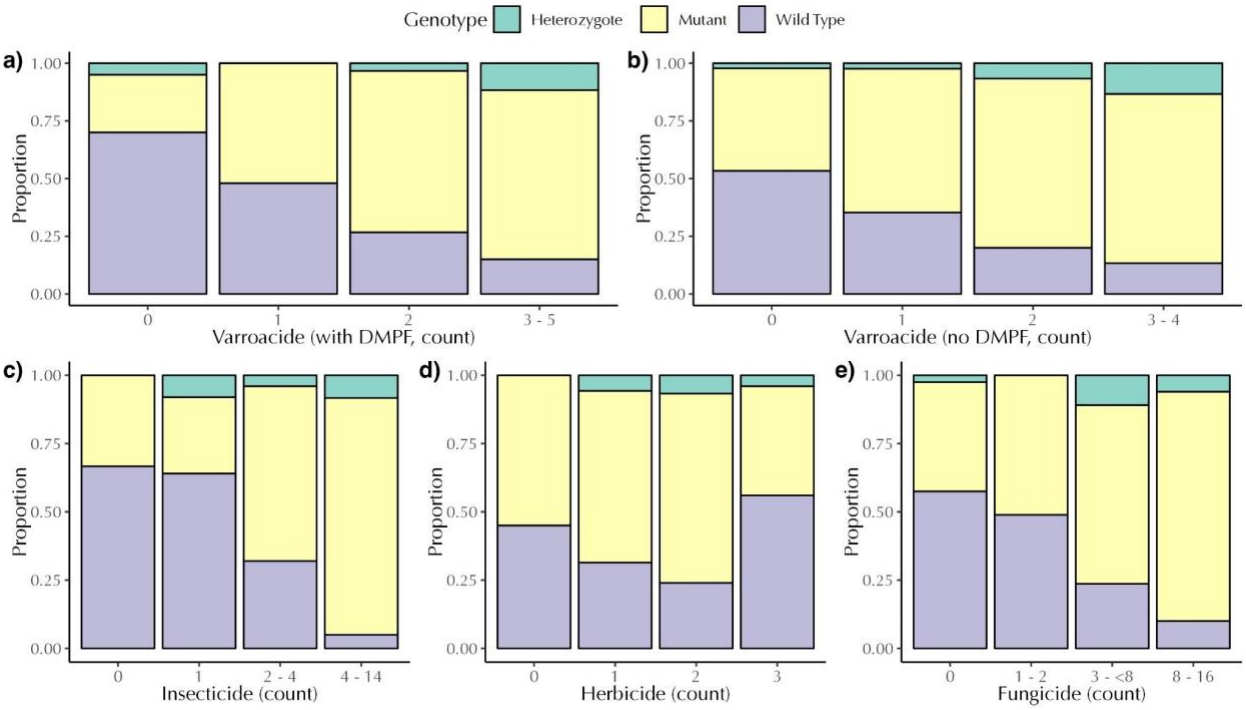

Figure 3. The proportion of homozygous Wildtype, heterozygous, or homozygous mutant for the Y215H mutation vs a.) varroacide count, b.) varroacide count data with DMPF removed, c) insecticide count data, d.) herbicide count data, & e.) fungicide count data divided into quartiles.

Table 1. Pesticides detected in the wax samples, individual pesticides, their class, and the number of detections (including trace), the prevalence found within the sample set, the number of detections excluding trace (n), the average concentration (Mean, SE, range) found within positive samples, and the average risk quotient score (mean, SE, range) posed by the pesticide in contaminated samples are presented

Pesticide	Class	Detections	Prevelence	Detections above LOQ	AVG PPB	Standard Error	Range	AVG RQ	RQ Standard Error
Acetamiprid	Insecticide	6	15.4%	0	Trace	0	0-Trace	Trace	0.000
Acetochlor	Herbicide	10	25.6%	1	130	0	130	0.02	6.384
Ametoctradin	Fungicide	2	5.1%	0	Trace	0	0-Trace	Trace	0.000
Azoxystrobin	Fungicide	23	59.0%	12	10.5	2.025	2-54	0.01	0.211
Bensulide	Herbicide	3	7.7%	2	9	0	9	0.05	0.000
Bifenazate	Insecticide	5	12.8%	5	78.2	14.17	15-268	1.18	161.711
Bifenthrin	Insecticide	2	5.1%	1	71	0	71	606.06	0.000
Boscalid	Fungicide	8	20.5%	5	10.2	0.82	6-21	0.01	0.011
Buprofezin	Insecticide	11	28.2%	8	19.75	3.41	2-77	0.01	22.270
Carbaryl	Insecticide	3	7.7%	0	Trace	0	0-Trace	Trace	0.000
Chlorantraniliprole	Insecticide	12	30.8%	4	22.75	1.58	15-41	0.29	0.002
Chlorothalonil	Fungicide	15	38.5%	3	223.67	43.01	12-636	0.21	0.016
Chlorthal-dimethyl	Herbicide	3	7.7%	1	4	0	4	0.01	0.452
Coumaphos	Varroacide	20	51.3%	15	64.67	12.32	7-243	1.40	0.000
Coumaphos oxon	Varroacide	16	41.0%	13	5.38	0.79	1-17	0.12	0.028
Cyprodinil	Fungicide	13	33.3%	13	13	0.91	3-24	0.01	0.078
DDE p,p'	Insecticide	3	7.7%	0	Trace	0	0-Trace	Trace	0.000
DEET	Insecticide	27	69.2%	6	26.17	2.43	13-57	0.02	0.000
Difenoconazole	Fungicide	5	12.8%	2	8	0.44	5-11	0.01	0.002
Diuron	herbicide	31	79.5%	11	24.45	3.50	6-78	0.02	0.039
DMPF	Varroacide	35	89.7%	30	2401.97	638.41	25-16700	12.30	0.147
Ethion	Insecticide	2	5.1%	0	Trace	0	0-Trace	Trace	0.139
Etoxazole	Insecticide	3	7.7%	2	3	0	2-4	0.00	0.000
Fenbuconazole	Fungicide	5	12.8%	1	44	0	44	1.02	0.000
Fenpyroximate	Insecticide	16	41.0%	10	42	9.32	4-227	0.02	0.000
Flonicamid	Insecticide	2	5.1%	0	Trace	0	0-Trace	Trace	0.000
Fludioxonil	Fungicide	2	5.1%	1	10	0	10	0.01	0.000
Fluopyram	Fungicide	20	51.3%	9	4.33	0.32	2-8	0.01	0.000
Flupyradifurone	Insecticide	2	5.1%	2	13	0	5-18	0.01	0.000
Fluvalinate	Varroacide	6	15.4%	5	252.6	38.75	73-769	7.49	0.000
Fluxapyroxad	Fungicide	14	35.9%	4	8.25	0.97	4-19	0.01	0.008
Hexazinone	Herbicide	11	28.2%	1	1	0	0-1	0.00	0.002
Hexythiazox	Insecticide	7	17.9%	4	3.25	0.24	2-6	0.00	0.000
Iprodione	Fungicide	10	25.6%	4	145.5	11.94	53-263	0.20	0.423
Metalaxyl	Fungicide	2	5.1%	0	Trace	0	0-Trace	Trace	0.000
Metconazole	Fungicide	1	2.6%	1	8	0	8	0.01	0.000
Methoxyfenozide	Insecticide	22	56.4%	16	18.63	3.12	3-84	0.02	0.000
Metolachlor	Herbicide	15	38.5%	2	27.5	0.22	26-29	0.03	0.000
Myclobutanil	Fungicide	2	5.1%	0	Trace	0	0-Trace	Trace	0.000
Novaluron	Insecticide	2	5.1%	2	11.5	0	7-16	0.01	0.000
Penthiopyrad	Fungicide	16	41.0%	5	5.4	0.50	3-10	0.00	0.000
Prometryn	Herbicide	1	2.6%	0	Trace	0	0-Trace	Trace	0.000
Propargite	Insecticide	29	74.4%	20	21.25	5.06	4-153	0.05	0.000
Propiconazole	Fungicide	13	33.3%	3	13	0.64	9-19	0.02	0.000
Pyraclostrobin	Fungicide	20	51.3%	18	14.44	1.63	3-40	0.02	0.000
Pyridaben	insecticide	4	10.3%	1	4	0	4	21.34	0.000
Pyrimethanil	Fungicide	17	43.6%	6	19.83	2.68	5-59	0.03	0.000
Pyriproxyfen	Insecticide	16	41.0%	0	Trace	0	0-Trace	Trace	0.000
Quinoxyfen	Fungicide	2	5.1%	1	3	0	3	0.00	0.000
Tebuconazole	Fungicide	14	35.9%	2	58.5	3.02	38-79	0.04	0.000
Tebufenozide	Insecticide	1	2.6%	1	14	0	14	0.01	0.000
Thymol	Varroacide	29	74.4%	26	4443.31	1993.72	27-70200	2.84	0.000
Triadimefon	Fungicide	6	15.4%	0	Trace	0	0-Trace	Trace	0.000
Triazophos	Insecticide	12	30.8%	4	3	0.10	3-4	0.01	0.000
Trifloxystrobin	Fungicide	27	69.2%	17	6.65	0.97	1-26	0.01	0.000

Pesticide concentration (ppb)

For all samples, the mean and standard error concentration of the sum of all quantifiable pesticide concentrations was 4,283.2±1,640.19 (ppb) with a median of 868.8 (ppb). Samples with higher concentrations of pesticides had higher frequencies of resistant alleles than samples with lower concentrations (Figure 1.b). When compared to the samples with lowest concentration of pesticides, the odds of finding resistance alleles were at least 2 times higher in mites originating from apiaries with higher levels of contamination (Figure 4). When categorized by class, the average sample had 80.09±29.89 ppb fungicide per samples, 9.5±5.42 ppb herbicide per sample, 47.51±13.51 ppb insecticide per sample, and 4130.08.15±1634.82 ppb varroacide per sample. Concentration data for each pesticide class were divided into quartiles and compared to the proportion of resistance alleles in varroa. Fungicide concentration samples in the second, third and fourth quartile were OR (95% CI) 4.5 (2.51-8.2), 4.2 (2.38-7.74), and 5.8 (3.0-11.85) respectively more likely to contain resistant varroa compared to the first quartile. As indicated by 95% CI overlapping 1, there was no evidence that mites from apiaries with higher herbicide concentrations had higher odds of having the Y215H mutation when comparing the first quartile to the second third and fourth OR (95%CI) 1.8 (0.89-4.09), 1.4 (0.77-2.44), and 1.8 (0.28-0.81) Increasing concentrations of insecticides in samples increased the odds of finding the Y215H mutation in varroa OR (95%CI) 4.0 (2.24-7.38), 5.2 (2.90-9.51), and 11.4 (5.59-25.16). Similarly, the odds of finding mites with Y215H mutation increased as the concentrations of varroacides increased, both with DMPF included when comparing the first quartile to the second third and fourth OR (95%CI) 4.7 (2.51-8.91), 3.6 (1.98-6.67), and 4.9 (2.72-8.90) and with DMPF excluded OR (95%CI) 2.9 (1.67-5.22), 2.0 (1.10-3.85), and 2.0 (1.12-3.58) more likely to contain resistant varroa compared to mites from apiaries in the first quartile.

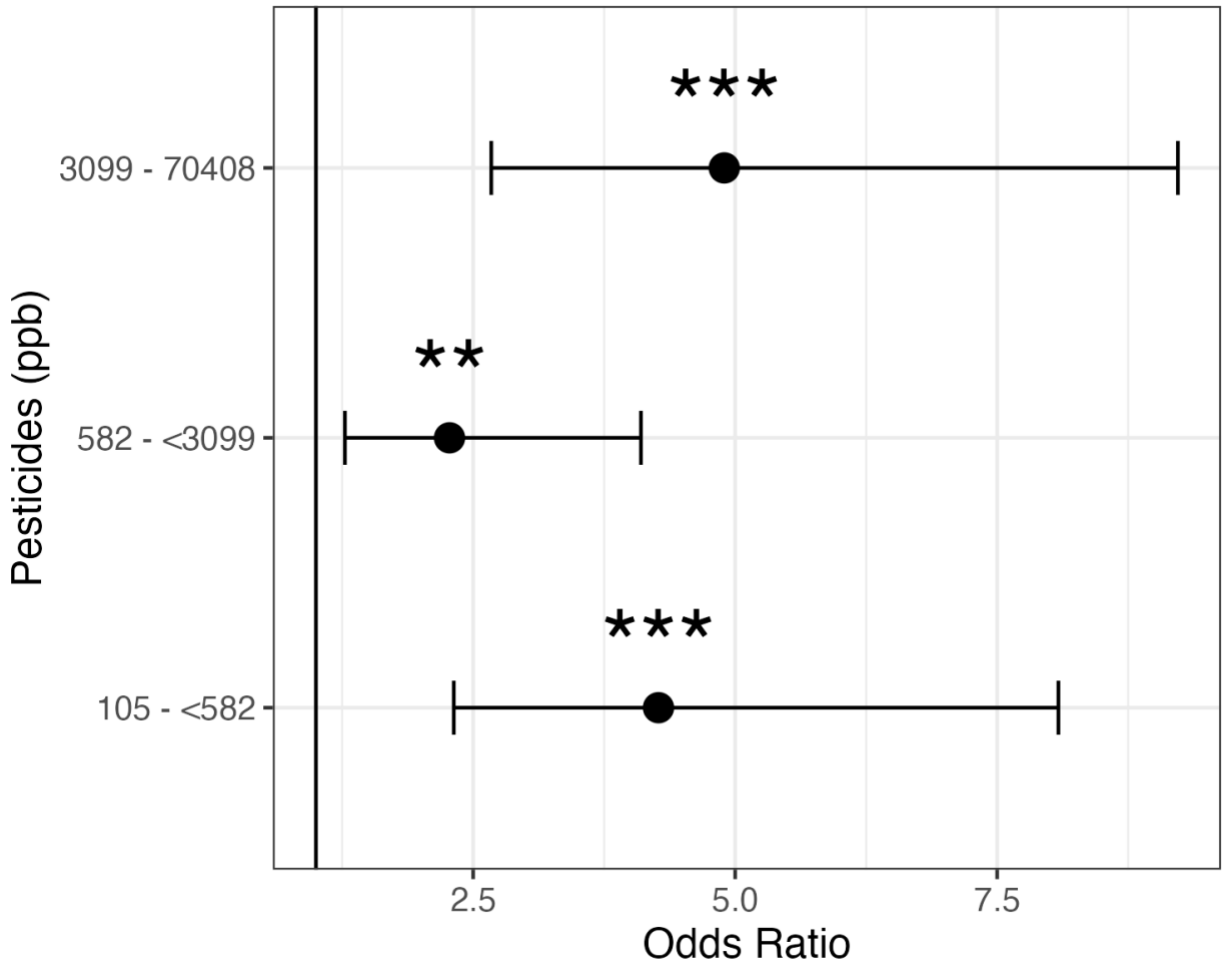

Figure 4. Odds ratio depicting the likelihood of encountering resistant mites in relation to the total concentration(ppb) of pesticides and metabolites in a sample. Statistically significant differences from the first quartile are marked with asterisks. Significance codes: 0***, 0.001**, 0.01*.

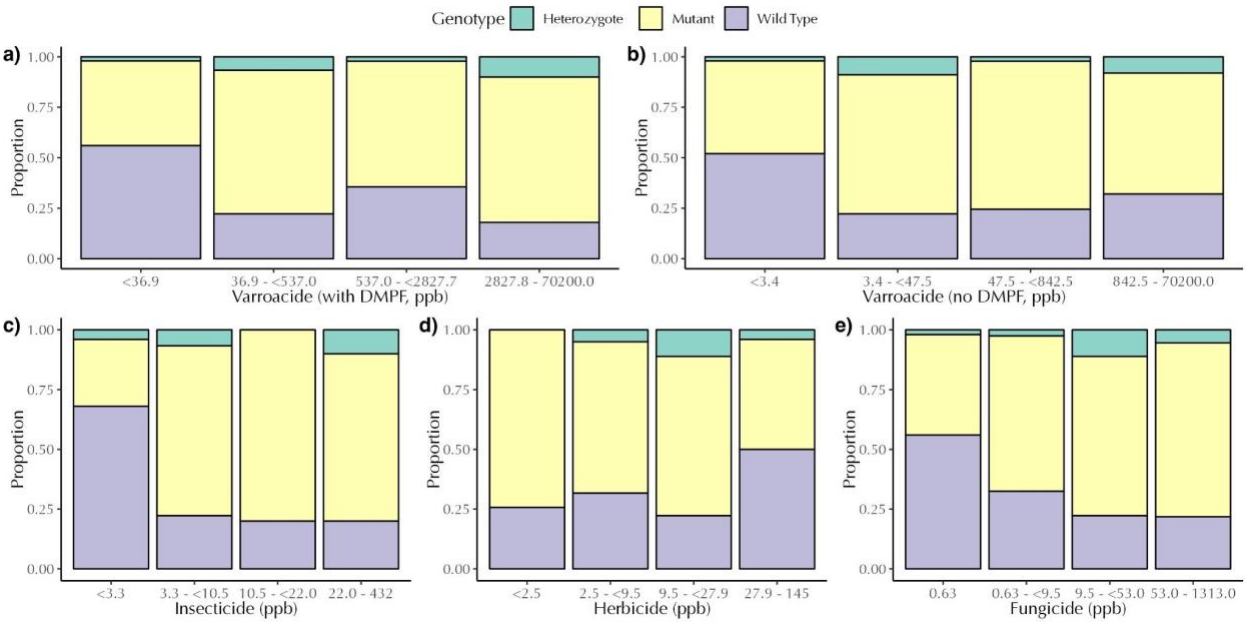

Figure 5. The proportion of mites that were homozygous wild type, heterozygous, or homozygous mutant for the Y215H mutation related to the concentration(ppb) when measured by pesticide class. a.) varroacide concentration including DMPF concentration data, b.) varroacide concentration with DMPF data removed from analysis, c.) insecticide concentration data, d.) herbicide concentration data, and e.) fungicide concentration data.

Within samples that had detectable levels of DMPF, the average concentration of DMPF was 2112.2 ±829.67ppb (Figure 6). When compared to the frequency of resistant mutations in the mites from apiaries with the absence/lowest levels of DMPF, the odds of encountering resistance genes increased as DMPF contamination increased (Figure 7).

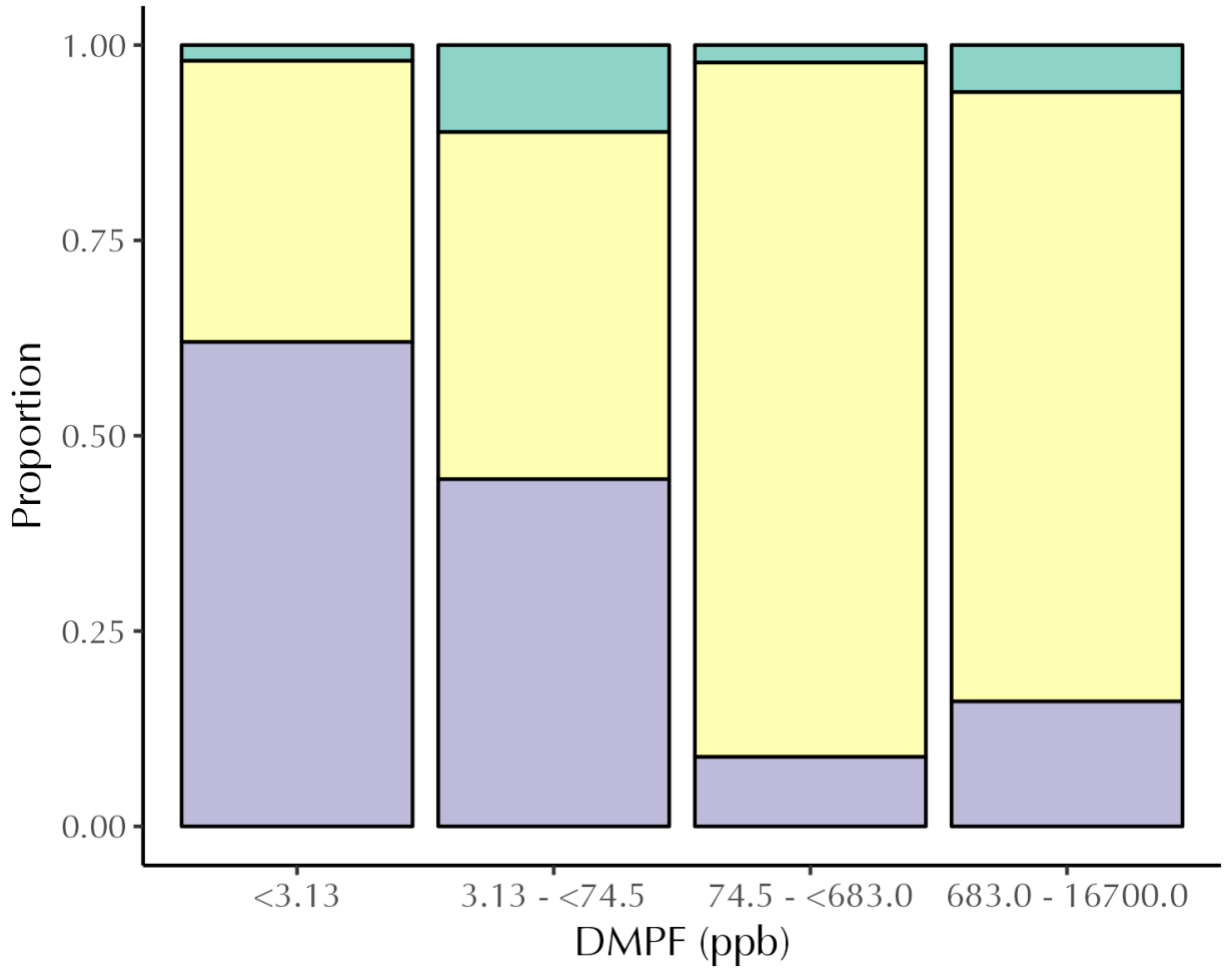

Figure 6. The proportion of mites that were homozygous wild type, heterozygous, or homozygous mutant for the Y215H mutation related to the concentration(ppb) of the amitraz metabolite DMPF.

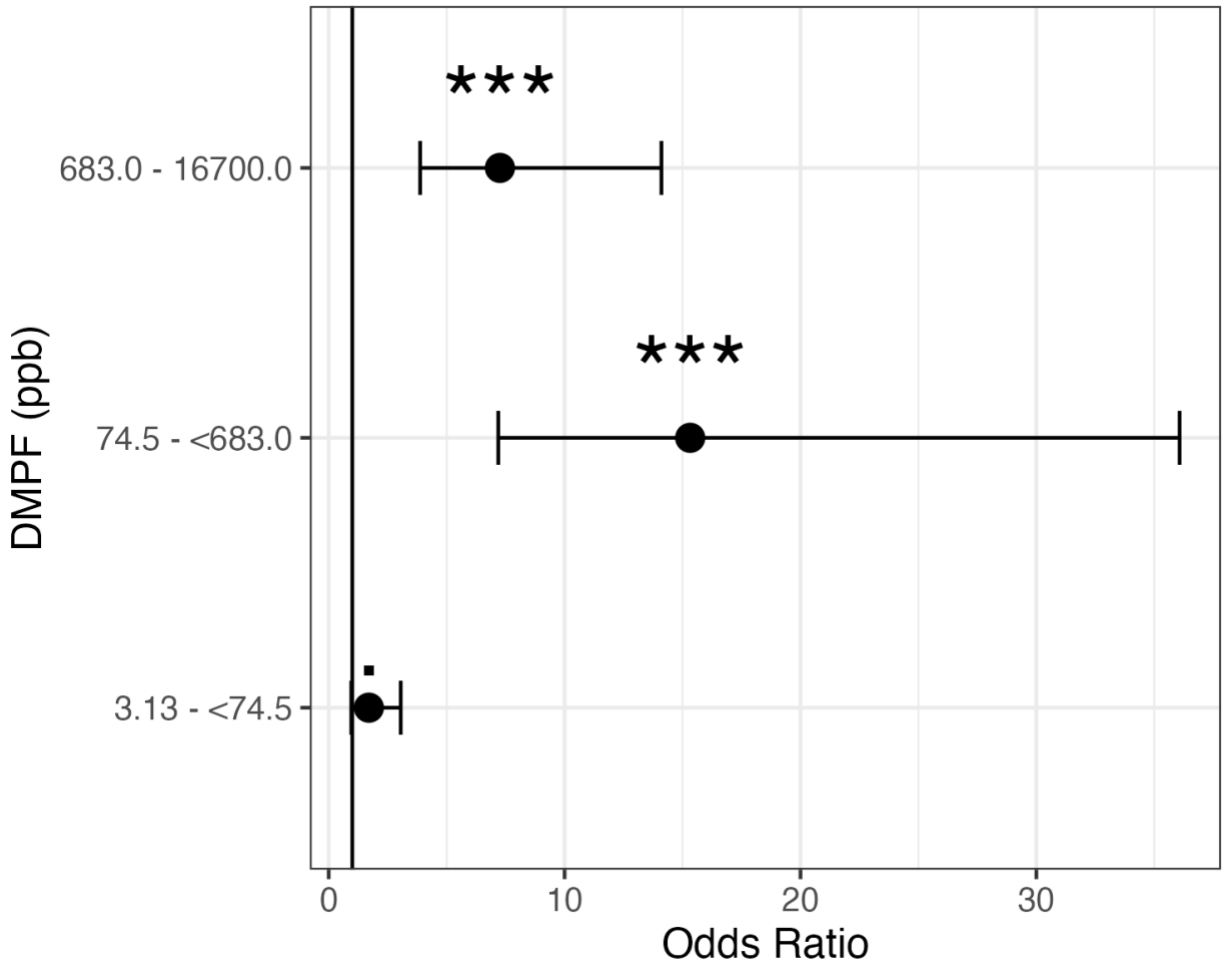

Figure 7. Odds ratio depicting the likelihood of encountering resistant mites in relation to the concentration (ppb) of DMPF in a sample from the second, third and fourth quartile compared to the first quartile. Significance codes: 0***, 0.001**, 0.01*.

Discussion

This study set out to determine the relationship between the occurrence of varroacide resistant genes in varroa and the residues of varroacides in the colonies from which the mites were collected. We only detected one mutation with enough frequency to test our hypothesis - this mutation (Y215H) was widespread, with 132 (of 195) mites found in 35 (of 39) apiaries. One hundred twenty one of the 132 were homozygous mutants, while 11 were heterozygous for

the mutant (and therefore phenotypically susceptible to treatment). Within hive conditions, amitraz has a half-life of between 12-55 hours (Korta et al. 2001) and so it was not screened for in our pesticide screening. When amitraz breaks down two major metabolites result. One of these metabolites, DMPF, is also toxic to mites (LC_{50} =0.497 µg/ml) (Morsy et al 2022) and was the most common product found in our samples. DMPF is similarly prevalent in other survey efforts (Lorenzo et al. 2019, Pohorecka et al. 2018). As expected, we found higher frequencies of resistant genes in mites that came from apiaries with detectable levels of DMPF, and that resistant genes generally occurred more frequently as the concentration of DMPF increases (Figure 6). When considering all varroacide classed pesticides we found that increasing numbers of varroacides in a sample, increasing and concentrations of varroacides were associated with increased detections of the Y215H mutation in the associated varroa mites. Considering the high prevalence of DMPF, and the high concentrations of DMPF in samples, we suspected DMPF was driving the relationship between varroacides and resistant genes.

As no other detected varroacides (or other detected pesticide) shared DMPFs mode of action (which targets the neuromodulator octopamine), there was no reason to suspect that other varroacides would select for mites carrying mutations conferring amitraz resistance. Changes in octopamine reception could affect varroa in many ways, changing the behavior development and physiology of mutant varroa. This possibility indicates further research into the Y215H mutation and the changes it has on varroa is needed to fully understand the impacts of this mutation. It is also possible that this mutation has become fixed in the US population. This would imply that the Y215H mutation does not have deleterious effects on mite fitness as has been documented for other pesticide conferring mutations (Kilot & Ghanim 2012). We expected that when DMPF positive detections were removed from the data set, the strong and consistent relationship

between varroacides and resistance genes would disappear when pesticide pressure was measured by count, or concentration. Surprisingly, we found that even with DMPF data removed, varroacide count and concentration data still had a positive relationship with the frequency of resistant Y125H mutations detected.

L925M and N87S resistance alleles

Not finding L925M gene and very few mites with N87S genes was unexpected, although this finding does complement findings in other agriculture systems like the tobacco bud worm (*Heliothis virescens)* (Fritz et al 2018, Fritz 2022). Past survey work using mites collected in 2013 found fluvalinate resistance in 97% of treated colonies and 10% in untreated colonies meaning we would have expected some positive results for the L925 mutation. We found only 5 apiaries had detectable levels of fluvalinate. Three of these 5 samples, were also the sites where the only 3 mites with N87S mutation were detected. These resistant genes occurred in colonies as heterozygous N87S mutants which are not phenotypically resistant. It is also worth noting that the three N87S mites were found in apiaries with high levels of pesticide detection. These detections loosely corroborate the findings of our more robust data set.

Significance for varroa management

Beekeeper mite management may provide some insight into this unexpected relationship between varroacides in general and the frequency of amitraz resistant mites. It is common for beekeepers, sometime after applying a treatment, to spot-check mite populations in order to ensure mite numbers are sufficiently controlled. If mite knock down is insufficient beekeepers may switch to a new treatment. It is possible that samples were collected from colonies whose mite populations were not successfully treated with amitraz due to resistant mites. The switch to

other products for varroa control could explain the high levels of these other varroacides, like the highly volatile thymol, seen in our samples.

Beekeeper management, however, does not explain the equally consistent and unexpected relationship between varroa gene mutation Y215H frequency and higher levels of exposures to insecticides (as measured by count, and concentration, (Figures 3c, 5c)), and fungicides measured by count (Figures 3e).

Combined, these results support the expected relationship between higher levels of contamination with an active ingredient (or its metabolite) and higher frequency of resistant genes. They also suggest that the active ingredient is not the only selection pressure, as increased amounts of other classes of pesticide also increases the likelihood of finding resistant alleles. Although a correlation between pesticides cannot be ruled out, and so the relationship may not be causal.

The impact of DMPF on resistance matches commonly seen trends in field evolved resistance (Hawkins et al. 2019). Beekeepers use amitraz frequently (Haber et al. 2019) and its breakdown product is often found within colony matrices (Pohorecka et al. 2018). This exposes mites to DMPF over their lifetime, which is an ideal environment to develop resistance (Hawkins et al. 2019). Persistent long term DMPF residues in wax can select for resistant alleles in mite population; however, the DMPF levels we detected in our samples could also have been residues from a recent treatment considering the long half-life (>8 months) of DMPF (Takata et al. 2020). While we could not discern between these two possibilities, future work in this area could have important implications for beekeepers as it may inform their brood comb management strategy (Underwood et al. 2023). If long term low levels of DMPF do not exert selection pressure on mites or otherwise negatively impact bee health, the need for expensive brood comb replacement

could be avoided. DMPF is known to be toxic to varroa, suggesting that DMPF itself is able to put selection pressure on mite populations (Takata et al. 2020), a suggestion our findings support. The question remains as to what concentration is needed to exert pressure. Considering that an increase in the number of pesticides detected, even at trace levels correlated with an increased frequency with which the Y215H gene was detected, our findings suggest that the presence of pesticide may be enough to influence allele frequency of resistance genes.

The association between mutation frequency and insecticides, fungicides and herbicide exposures suggest that the Y215H mutation can become stable in populations without varroacide pressure, suggesting that Y215H does not adversely affect mites. The L925M mutation appears to have the more typical loss of fitness associated with resistant mutations. Without fluvalinate pressure the L925M mutants are selected against due to decreased mite fitness (González-Cabrera et al. 2013). The high prevalence of the Y215H mutation, even in unexposed populations, suggests that the mutation may confer some other benefit to mites. One alternative explanation would involve the mite bomb hypothesis dispersing resistant mites across the landscape to colonies without varroacide pressure (Kulhanek et al. 2021). This would require there to be no loss of fitness for Y215H mites in the absence of DMPF. Another alternative explanation may be the uniform structure of the U.S. mite population. In part this is facilitated by the reproductive strategy of mites, which often involves sibling mating resulting in remarkably uniform genetic makeup of mites within an apiary (Roth et al. 2020). What genetic differences there are in mite populations are also equalized by the nature of commercial beekeeping in the U.S., the large migratory events required to pollinate crops, and the shipping of bees across the country, would provide mechanisms for favorable genes to quickly be spread and be maintained in the population.

Our results have important implications for beekeepers. Product rotation and management, need to monitor during treatment, and determining pesticide levels in wax can help flag potential resistant problems associated with the Y215H mutation. One possible long-term solution is breeding mite resistant bees to a point that these honey bee stock no longer require varroacide. In the meantime, beekeepers should consider an IPM approach to chemical treatment for varroa and consider non-chemical management options where possible.

The presence of the Y215H mutation within honey bees in the U.S. and increasing treatment failures of amitraz will negatively impact beekeepers who are reliant on amitraz for varroa control as these mutant varroa are linked to amitraz treatment failures in the U.S. (Rinkevich 2020). Resistance to DMPF (and its parent amitraz) indicates a longer break in amitraz use could help to further reduce the selection pressure for resistant mites. However, the association between resistant allele frequency and non-varroacide contamination seems to indicate an extended break and new, contaminant free comb may be needed for this rotation to be effective, if at all. Our finding that resistant alleles seem to be prevalent even without amitraz exposure, seems different from trends found for the fluvalinate resistance meaning further research and experimentation may be needed. Combining chemical and non-chemical treatments to manage varroa can also help beekeepers decrease the proportion of resistant mites in their apiaries.

Field evolved resistance in varroa poses problems for beekeepers that will require more targeted and informed varroa management practices to ensure colony health. One such method is the growing body of work supporting the use of alternating active ingredient treatments for varroa control (Giacobino et al. 2016, Haber et al. 2019 Traynor et al. 2020). By changing the chemical treatment used, beekeepers could shift selective pressure to select against amitraz

resistant varroa, reducing their proportion in a population. In addition to rotating chemical treatments, brood breaks and mechanical control can be used as part of treatments to reduce varroa load in an apiary (Warner et al. 2023, Haber et al. 2019). High dose rotating treatments are also an option; these involve using high levels of treatment and based on observations of decreased efficacy, seasonal timing, and alternating between chemical treatments (González-Cabrera et al. 2016). In addition to informed treatment, further research could increase our understanding of varroa resistance and pesticide pressure on honey bees.

Collecting management data from participants would have provided more information about how both chemical and non-chemical treatments affect the proportion of resistance mites found in apiaries. Collecting paired brood wax samples and varroa at multiple points throughout a season would also provide trend data for seasonal changes in pesticide pressure and the impact these changes have on resistance levels within an apiary. Taking samples from individual hives rather than apiaries would also provide more granularity in the relation between specific pesticide conditions and varroa resistance.

Conclusion

This project set out to investigate the relationship between pesticides residues within beeswax and the frequency of resistance alleles in varroa mites. Wax samples were analyzed for pesticides residues and varroa were genotyped for three different known resistance alleles. When analyzed by pesticide count and concentration, not only did the resisted pesticide show a positive relationship with increased presence of resistant alleles, but pesticides in different classes that do not share a mode of action with amitraz (and DMPF) also showed the same relationship. These

findings have important long-term implications for beekeepers, who will need to adopt new and more specialized control systems for varroa in the near future.

Supplemental table 1

Supplemental table 1. Pesticide sample ID, State of origin, pesticide detections, total risk quotient, total concentration (ppb), and the allele breakdown of the 215-resistance allele in the varroa from each sample.

Sample ID	State	Pesticide detections	total Hazard	total ppb	homozygous 215mut	homozygous 215WT	heterozygous 215WT/215mut
CA-02	California	32	43.06	3429	5	0	0
CA-03	California	38	152.63	3108	5	0	0
CA-05	California	28	1214.65	12390	5	0	0
CA-07	California	26	287.17	20818	4	0	1
CO-01	Colorado	14	24.18	4399	4	0	1
CO-02	Colorado	15	13.3	2018	1	3	1
DC-17	Washington DC	2	0.56	6	2	3	0
DC-18	Washington DC	6	1.67	46	3	2	0
DC-19	Washington DC	2	0.2	744	2	3	0
DC-20	Washington DC	3	4.35	66	2	3	0
DC-21	Washington DC	8	1.33	27	1	4	0
DC-22	Washington DC	4	47.98	1303	4	1	0
DC-23	Washington DC	3	0.56	45	1	4	0
DC-24	Washington DC	4	4.84	892	2	3	0
DE-18	Delaware	4	0.82	135	0	4	1
DE-19	Delaware	1	0	6	0	5	0
DE-20	Delaware	8	353.96	70419	0	5	0
DE-21	Delaware	15	2.89	160	0	2	3
DE-22	Delaware	6	10.52	1342	2	3	0
DE-23	Delaware	4	0.39	0	0	5	0
MI-12	Michigan	23	209.74	5575	3	1	1
MI-13	Michigan	16	60.36	1969	5	0	0
MI-14	Michigan	15	8.42	243	4	1	0
MN-15	Minnesota	12	64.93	878	4	1	0
MN-16	Minnesota	11	0.43	9	5	0	0
MN-19	Minnesota	11	7.07	135	5	0	0
NM-01	New Mexico	6	37.23	834	0	5	0
NM-02	New Mexico	11	2	132	3	2	0
NM-03	New Mexico	13	633.81	15963	5	0	0
NM-05	New Mexico	8	30.33	4047	2	2	1
NM-06	New Mexico	7	669.43	16703	4	1	0
NM-07	New Mexico	7	50.55	1706	5	0	0
NV-18	Nevada	10	136.73	2924	4	0	1
WA-07	Washington	14	0.44	43	5	0	0
WA-08	Washington	21	34.38	305	5	0	0
WA-10	Washington	9	7	169	5	0	0
WA-11	Washington	8	12.17	287	5	0	0
WA-13	Washington	10	70.24	153	5	0	0

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